THE ROLE OF CRUMBS PROTEINS IN MAMMALIAN GASTRULATION

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Nitya Ramkumar

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THE ROLE OF CRUMBS PROTEINS IN MAMMALIAN GASTRULATION

Nitya Ramkumar, PhD

Cornell University, 2015

Crumbs, a transmembrane protein with a short cytoplasmic domain and very large extracellular domain, was identified as an apical polarity determinant in *Drosophila* that is required for epithelial integrity. In mammals the Crumbs family consists of three members, *Crumbs1, Crumbs2* and *Crumbs3*. Here I will show that mammalian Crumbs family is not essential for establishing epithelial polarity in the early mouse embryo and that the earliest function of the Crumbs family in mammals is the regulation of gastrulation by Crumbs2.

Mutations in human *CRUMBS1* have been associated with Retinitis pigmentosa and retinal degeneration. Most mutations lie in the extracellular domain of Crumbs, whose function still remains unclear. Here I show that the extracellular domain of Crumbs2 is O-glucosylated by an enzyme Protein O-glucosyltransferse 1, which regulates its membrane localization. The apical membrane localization of Crumbs2 is essential for its function in mammalian gastrulation.

The gastrulation phenotype in *Crumbs2* mutants results in the accumulation of cells at the primitive streak. Although Crumbs proteins are expected to be required to maintain the apical domain of the cell, mammalian Crumbs2 is required for cells to lose apical adherens junctions and to facilitate cell delamination during gastrulation epithelial-to-mesenchymal transition. The requirement of Crumbs2 in epithelial organization and cell delamination at the primitive streak is locally non-cell autonomous, as *Crumbs2* mutant cells can delaminate, migrate away and be incorporated into all lineages when surrounded by wild-type cells. Further, we find

that Crumbs2 is required in the epiblast adjacent to the primitive streak to maintain epithelial integrity, but only when neighboring cells are undergoing cell rearrangements such as the gastrulation EMT, as the local disorganization of cells in the epiblast does not occur in the absence of the primitive streak. Thus I propose that Crumbs2 is part of a global system that maintains epiblast integrity while cells delaminate during the EMT in mammalian gastrulation.

BIOGRAPHICAL SKETCH

Nitya Ramkumar was born August 3, 1985 in New Delhi, India to parents Sumati and Ramkumar and elder sister Pavitra. As a child she was extremely curious and loved questioning the way things worked. She developed an early liking for Mathematics and Science. With her elder sister as her partner in crime, together they experimented with everything from household electronics to their dad's instrumental toolkit- him being an instrumental engineer. Her scientific temperament was recognized early on at her school where she won awards at the annual science fairs. She went on to pursue an undergraduate degree in Biotechnology at Vellore Institute of Technology. Following this she worked in the research lab of Dr. Shubha Tole where she explored factors that affect post eclosion behavior in Drosophila. In the fall of 2008 she moved to New York to enter the Biochemistry, Cell and Molecular biology program at the Weill Cornell Graduate School of Medical Sciences. During her rotations she became fascinated with the fundamental questions in developmental biology and decided to join the laboratory of Dr. Kathryn Anderson wherein she worked on the role of Crumbs proteins in regulating mammalian gastrulation.

After graduating from Cornell she will be moving to London to work with Dr. Buzz Baum on cell division dynamics in Drosophila epithelial tissue.

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I am greatly indebted to my family for all their love and support. My grandmother, who is 78, continues to work at the Cancer Research Institute,

Chennai and has been my inspiration to be persistent in research. My parents have always supported me in all my endeavors and continue to keep my spirits high especially in trying times. I couldn't thank them more for being such great role models. I am thankful to my sister and jiju who helped me during my move to New York and always made me feel at home. Their optimism was contagious and motivating and kept me in sight of my goals. I am grateful to my husband Hemant for being very patient, tolerant, thoughtful and caring and especially thankful to him for making numerous trips across the Atlantic during the course of my graduate school. He knows exactly how to cheer me up and makes sure that no matter what there is always a reason to smile.

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1.1 Morphogenesis of the mouse embryo

Following implantation, by embryonic day E5.5, the mouse embryo organizes itself into a cup shaped structure consisting of two layers of cells, the epiblast (ectoderm) and visceral endoderm. By E6.25, the process of gastrulation begins, which introduces a new cell layer called mesoderm, sandwiched between the existing two and transforms the embryo into three layers of cells. Like most animal embryos, mouse embryos are triploblastic: they lay down the body plan with three germ layers and each germ layer gives rise to specific tissues in the adult as determined by lineage tracing experiments. The ectoderm gives rise to the brain, skin and spinal cord, the endoderm gives rise to internal organs like gut and digestive system, and mesoderm layer gives rise to bones and musculature and circulatory system in the adult. The process of establishing the germ layers begins at E6.25 and occurs continuously for a few days. Simultaneously, the embryo grows in size and each germ layer begins differentiating into the different structures in the embryo. For example, the neural epithelium begins to close and form the neural tube, which will eventually give rise to the brain and spinal cord. The initial process of laying the foundation of the body plan and gastrulation seems to be conserved in other mammals, including human embryos, and at least some of the signaling pathways regulating these processes are also conserved in human embryos (Ferrer-Vaquer and Hadjantonakis, 2013; Herion et al., 2014).

Gastrulation is a highly regulated process and occurs at a structure on the posterior side of the embryo called the primitive streak. The primitive streak is a

hub of signaling pathways. It is a transient structure present during the development of avian, reptilian and mammalian embryos. In the absence of a primitive streak, mouse embryos fail to form three germ layers and remain as two layers of cells (Liu et al., 1999).

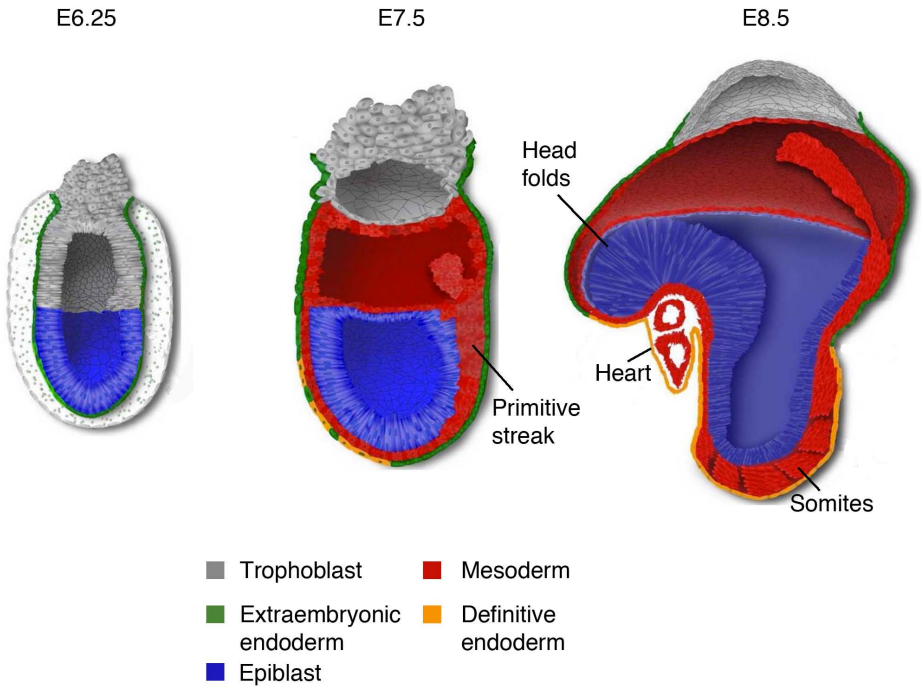


Figure 1.1 Morphogenesis of the mouse embryo
Schematic representation of the mouse embryo from pre-gastrulation stage E6.25 to E8.5. While the three germ layers are being specified, the embryo increases in size and simultaneously begins to differentiate the germ layers into structures like neural tube, heart and somites. Adapted from (Nowotschin and Hadjantonakis, 2010).

1.2. Epithelial to Mesenchymal transition

Epithelial cells act as a barrier between the outside and the embryo. These cells are polarized with well defined apical and basolateral domains maintained by cell junctions. Prior to gastrulation, the mouse embryo consists of two epithelial cell layers, the epiblast and the visceral endoderm. These layers share a common basement membrane and the apical side of the epiblast faces the interior of the embryo while that of the endoderm faces the outside. Mammalian gastrulation consists of a highly regulated epithelial-to-mesenchymal transition (EMT) that transforms the two-layered embryo into three layers. EMT is a multistep process, wherein well-polarized epithelial cells lose their basement membrane and their cell junctions and adopt migratory characteristics. Following EMT, these cells can remain mesenchymal and populate the embryo or undergo a reverse process called MET and become epithelial again and be incorporated into definitive endoderm or somites. EMT is vital for various developmental processes such as gastrulation, neural crest development and cardiac valve formation (Lim and Thiery, 2012). In adults, EMT is important for wound healing and has been implicated as a mechanism for cancer metastasis. Tumor cells undergo EMT, which then allows them to disseminate and colonize other areas of the body (Iwatsuki et al., 2010). Recently EMT has been implicated in bestowing stem cell line properties to cells (Mani et al., 2008). The handful of transcription factors that poise the cell to do an EMT can also induce the ability of the cell to adopt different fates following EMT (Puisieux et al., 2014). This is particularly true of the gastrulation EMT. As cells exit from the streak, they progressively adopt different fates. The first cells to exit from the streak give rise to extra-embryonic, cardiac and cranial mesoderm, as well as definitive endoderm, while later cells give rise to lateral plate and then paraxial mesoderm (Kinder et al., 1999). During

gastrulation, the fate of these cells is remarkably plastic and heterotopic transplantation reveals that cells adopt new fates depending upon their new local environment (Tam et al., 1997). This suggests that timing and location are very important for cell fate determination during mammalian gastrulation.

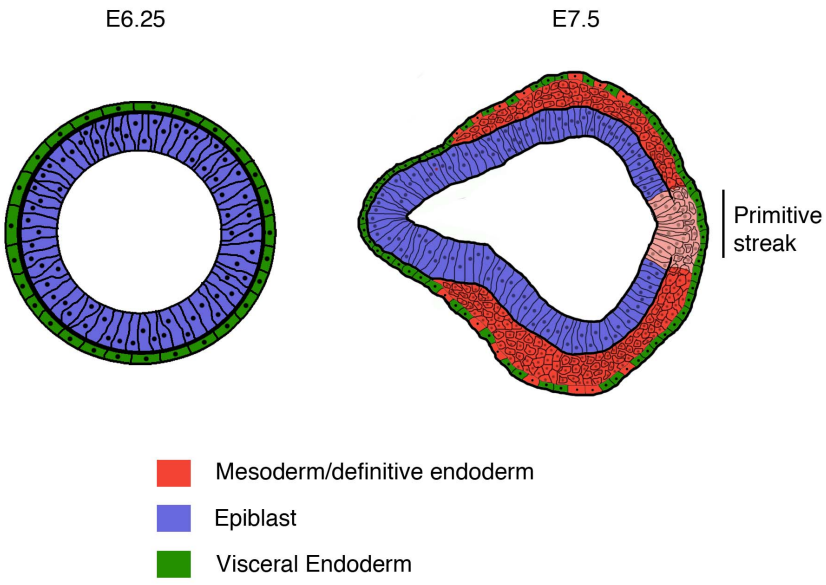


Figure 1.2 Epithelial to mesenchymal transition during gastrulation. Transformation of two epithelial cell layers to three layers with the formation of mesenchymal cell layer. The radial symmetry of the embryo is broken down by formation of the streak in the posterior side.

Because of its universal importance, the process of EMT and its regulators have been extensively investigated. Novel regulators of EMT are being discovered with screens in cell lines and mouse. Mutations in EMT regulators have been associated with many cancers (Tania et al., 2014). However, very little is known about their biological significance or their mechanism *in vivo*. An additional layer

of complexity is that different players regulate this multistep process depending on the context. Recent investigations are beginning to shed light on the complexity of EMT. Conventionally, it was thought that loss of adherens junctions promotes EMT and leads to more aggressive tumors as cells could disseminate easily. However, recently it was shown that cell junctions are required for mammary tumor cell dissemination (Shamir et al., 2014). These new findings suggest that our understanding of this complex process is still in its nascent stages.

1.2.1 Basement membrane breakdown and matrix reorganization

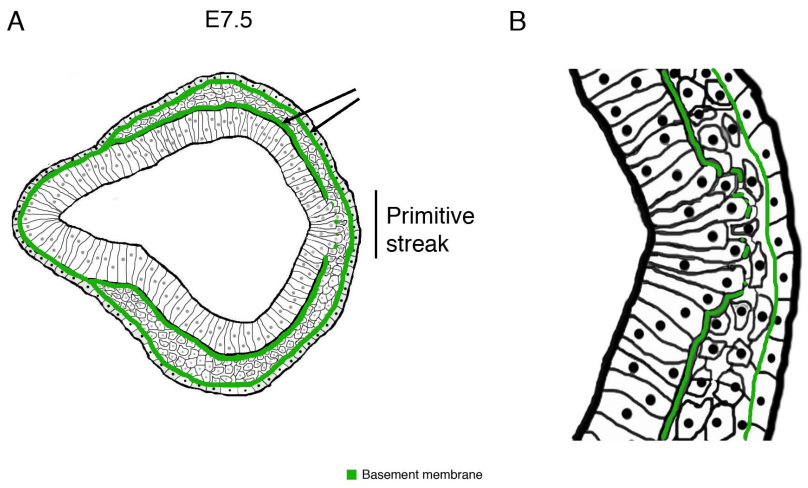


Figure 1.3 Localized breakdown of basement membrane at the primitive streak The basement membrane is continuous throughout the epiblast and broken down locally at the primitive streak (A). Notice the formation of the second basement membrane as gastrulation proceeds (arrows). High magnification view of basement membrane breakdown at the primitive streak (B).

The two epithelial cell layers of the early post-implantation mouse embryo share a common basement membrane. Basement membrane is a layer of highly cross-

linked extracellular matrix. The formation of a mesoderm cell layer transforms the embryo from containing one basement membrane to two basement membranes with the mesoderm layer sandwiched in-between (Viotti et al., 2014). The first event of EMT is the localized breakdown of basement membrane specifically at the primitive streak, which allows the cells to move through the extracellular matrix following delamination. The breakdown of basement membrane is tightly linked to the formation of primitive streak. Mouse mutants that fail to form a primitive streak do not have breaks in basement membrane (Liu et al., 1999).

Studies in chick have shown that local down-regulation of RhoA activity and microtubule dynamics regulate basement membrane breakdown. Failure to down-regulate basal RhoA leads to retention of basement membrane in epiblast cells at the streak (Nakaya et al., 2013; Nakaya et al., 2008). The disengagement of CLASPs (CLIP-associated proteins), microtubule plus end tracking proteins, with Dystroglycan, a component of basement membrane regulates microtubule reorganization at the amniote primitive streak and promotes basement membrane breakdown. The upstream regulators of RhoA and CLASPs remain unknown.

The local breakdown of basement membrane is thought to be the first step of gastrulation EMT. As the primitive streak elongates along the proximal-distal axis at the onset of gastrulation (early streak to late streak), so does the breakdown of basement membrane (Williams et al., 2012). Gastrulation mutants that fail to

down-regulate *E-cadherin* such as *Snail1* still have a break in the basement membrane (Carver et al., 2001). Mouse mutant for *fibronectin*, a component of the basement membrane, implant and develop until mid-gestation and the defects in these embryos correspond to a dearth of mesoderm-derived structures (George et al., 1993). However, even in *fibronectin* null embryos, there is a local breakdown of basement membrane (George et al., 1993). Integrin, another component of the basement membrane matrix, is also important for mammalian gastrulation. Mouse embryos deficient of *integrin α5-β1*, a primary receptor for fibronectin, and others affecting integrin-based signaling like *Paxillin* and *Focal adhesion kinase* have defects similar to *fibronectin* mutants in mesoderm-derived structures (Furuta et al., 1995; Giros et al., 2011; Hagel et al., 2002). The roles of these proteins in gastrulation were linked to the migration of nascent mesoderm cells, as fibroblasts obtained from *Paxillin* and *Focal adhesion kinase* mutants failed to form normal focal adhesions and had reduced cell migration (Hagel et al., 2002; Ilic et al., 1995). A role of fibronectin-based integrin signaling in maintaining epiblast integrity has not been investigated.

1.2.2. Regulation of E-cadherin expression

A major characteristic and requisite for EMT is the down-regulation of adherens junctions (Burdsal et al., 1993). The adherens junction is a complex of proteins that defines the apical region of epithelial cell-cell contacts and couples the cell junctions to the actin cytoskeleton. E-cadherin is a transmembrane protein that mediates calcium-dependent cell-cell adhesion. E-cadherin is essential for development, as mouse embryos die before implantation in the absence of zygotic *E-cadherin* (Riethmacher et al., 1995). Epiblast cells strongly express E-cadherin. The down-regulation of E-cadherin at the primitive streak is important

for cells to migrate as cells accumulate at the streak in mutants that fail to down-regulate E-cadherin (Ciruna and Rossant, 2001; Sun et al., 1999). The down-regulation of E-cadherin is gradual as epithelial cells at the streak still retain E-cadherin expression and initiate the down-regulation only as they leave the epithelium (Figure 1.4). The expression and levels of E-cadherin are regulated both transcriptionally and post-translationally (Arnold et al., 2008; Carver et al., 2001; Zohn et al., 2006).

The transcription factor *Snail1* is a major regulator of E-cadherin expression. *Snail1* is the founding member of the zinc-finger transcription factor family Snail, which in mammals includes *Slug (Snail2)* and *Smuc (Snail3)*. Snail1 directly represses the transcription of E-cadherin by binding to the E-box domains in its promoter region (Batlle et al., 2000; Cano et al., 2000). In addition to E-cadherin, Snail1 also represses the transcription of Claudins and Occludins, tight junction proteins, and promotes the expression of Vimentin and Fibronectin, mesoderm proteins. Among the members of the Snail family in mice, only *Snail1* is expressed in the primitive streak and migrating mesoderm cells during gastrulation (Smith et al., 1992). Mouse mutants for *Slug1* and *Smuc* are viable and fertile. *Snail1* mutants have disrupted gastrulation. Cells delaminate at the streak but these cells still retain E-cadherin expression and polarity and remain epithelial (Carver et al., 2001). This suggests that down-regulation of E-cadherin is not essential for cell delamination and formation of a third cell layer. Besides these interesting observations, the gastrulation defect in *Snail1* mutants remains poorly characterized.

The signaling networks that regulate the streak also control the expression of Snail1. Fgf signaling is thought to regulate *Snail1* expression, as mouse *Fgfr1* mutants fail to express Snail1 as seen by in situ hybridization. *Fgfr1* mutant cells retain *E-cadherin* expression and therefore accumulate at the primitive streak (Ciruna and Rossant, 2001). Mouse mutants for *Fgf8*, its ligand, have a similar phenotype wherein cells fail to migrate following EMT and retain E-cadherin expression (Sun et al., 1999).

In addition to *Snail1*, the transcription factor *Eomesodermin* was shown to affect E-cadherin levels. Conditional deletion of *Eomesodermin* in the epiblast results in a gastrulation phenotype with cells accumulated at the primitive streak. These cells fail to down-regulate E-cadherin protein and RNA expression, despite normal Fgf signaling and *Snail1* expression at the streak (Arnold et al., 2008). The targets of *Eomesodermin*, *Mesoderm Posterior 1 and 2* (*Mesp1* and *Mesp2*) also have roles in mammalian gastrulation (Costello et al., 2011). Mouse mutants for both *Mesp1* and *Mesp2* have a gastrulation phenotype similar to conditional deletion of *Eomesodermin* (Kitajima et al., 2000). The mechanism of their action remains unknown.

The levels of E-cadherin are also regulated post transcriptionally. *p38 MAP kinase* and *p38 interacting protein* are required for E-cadherin protein down-regulation. Mouse mutants for *p38* and *p38IP*, have normal *Snail1* expression at the streak, but cells fail to down-regulate E-cadherin and accumulate at the streak (Zohn et al., 2006). *p38* is thought to act downstream of *Map4k4* (*NIK*), as mice lacking *NIK* have cell accumulations at the streak and fail to upregulate p38 expression (Zohn et al., 2006).

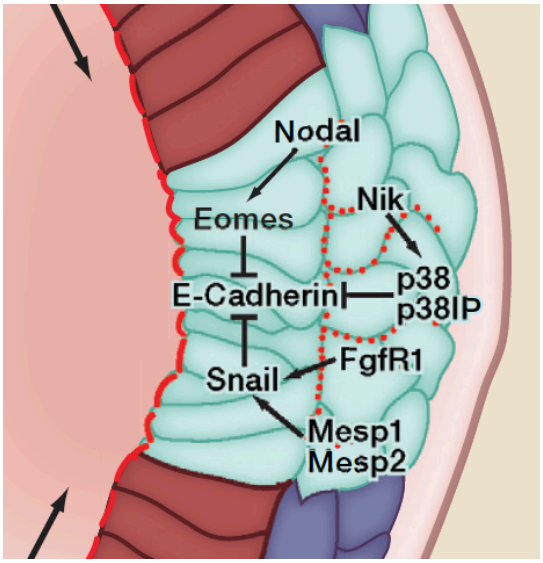


Figure 1.4 Down-regulation of E-cadherin expression at the primitive streak Transcriptional levels of *E-cadherin* are regulated by *Snail1* and *Eomesodermin*, whose levels are regulated by FGF signaling and *Nodal* respectively. Post-transcriptional levels of E-cadherin are regulated by *p38* and *p38IP*.

The other transcription factors known to regulate E-cadherin from extensive studies in cell lines are *Twist* and the *ZEBs*. *Twist1*, a basic helix-loop-helix transcription factor is not expressed at the streak, but is expressed later in the lateral mesoderm and then continues to be expressed in the anterior cranial mesenchyme. Mouse embryos lacking *Twist1* have less cranial mesoderm and fail to close the neural tube (Chen and Behringer, 1995). However, they do not have defects in the primitive streak. The *Zeb1* and *Zeb2* double mutant embryos also do not have a gastrulation phenotype, indicating that these proteins probably do not play an essential role during gastrulation EMT (Miyoshi et al., 2006).

A number of other proteins have been implicated in the gastrulation EMT, however their roles remain unclear. *Geminin*, a multifunctional protein involved in cell-cycle progression, had a negative correlation with *E-cadherin* expression (Emmett and O'Shea, 2012). Mice lacking *Yin-Yang1*, a polycomb group protein, in embryonic tissues had a severe gastrulation phenotype. The cells at the streak failed to down regulate E-cadherin and undergo EMT. It is thought to function by regulating Nodal signaling (Trask et al., 2012). The levels of Nodal are very important for gastrulation EMT. Several mutants that directly or indirectly regulate Nodal expression have a gastrulation phenotype. Mice lacking *Mixl1* have upregulated levels of Nodal signaling and an accumulation of mesoderm cells at the primitive streak (Hart et al., 2002). *Cripto* acts both as a cofactor and ligand for Nodal signaling. Conditional deletion of *Cripto* in the epiblast leads to accumulation of mesoderm like cells at the primitive streak. In addition to Nodal signaling, FGF signaling and p38 MAP Kinase pathway are also affected in these mutants (Jin and Ding, 2013).

1.2.3. Cytoskeletal reorganization

The EMT transforms polarized epithelial cells into migratory mesoderm cells. Epithelial and mesoderm cells have vastly different cytoskeletal organization to suit their respective functions. The epiblast cells have actin and myosin enriched at the apical side of the cells. When epiblast cells arrive at the primitive streak, they adopt a bottleneck shape. They constrict apically, and therefore reduce the apical cell surface area, following which the cells delaminate (Nakaya and Sheng, 2009). The requirement of apical constriction for cell delamination remains unclear. It is thought to help in maintaining epithelial stability, as a smaller gap in

the epithelium is easier to fill than a bigger one. Following delamination, or simultaneously with it, the cells acquire migratory ability for which their actin cytoskeleton needs to be dramatically reorganized to facilitate formation of dynamic structures like filopodia and lamellopodia.

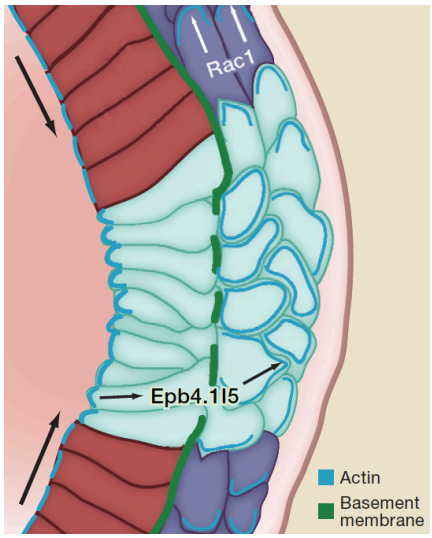


Figure 1.5 Cytoskeletal re-organization during EMT
Actin is enriched on the apical side of the epiblast cells and reorganized by Epb4.1l5 during EMT. Rac1 regulates actin dynamics to facilitate cell migration.

Mutations in proteins that affect the actin cytoskeleton can affect the gastrulation EMT directly or affect the migration of cells following EMT. *Wiskott-Aldrich syndrome protein (WASP)* and *WASP-family verprolin-homologous protein (WAVE)* family proteins are major actin reorganizers required for the formation of branched actin networks throughout eukaryotes (Kurisu and Takenawa, 2009). *Nap1* is a member of the WAVE complex. In mutants lacking *Nap1*, the WAVE complex is disrupted and cells accumulate at the streak. These cells undergo

normal EMT but fail to reorganize their actin cytoskeleton to promote cell migration (Rakeman and Anderson, 2006). Among the RhoGTPases, *Rac1* was shown to be important for cell migration during gastrulation. Conditional deletion of *Rac1* in the epiblast impairs the migration of mesoderm cells following EMT. *Rac1* is thought to affect actin dynamics upstream of the WAVE complex (Migeotte et al., 2011). *β-pix* is a Rho-GEF, which controls the spatial localization of Rac1 activation. Conditional deletion of *β-pix* in embryonic tissues causes a gastrulation phenotype with reduction in mesoderm-derived tissues (Omelchenko et al., 2014).

In addition to facilitating cell migration, cells that cannot reorganize the cellular cytoskeleton can be stuck in an intermediate stage of EMT. *Epb4.1l5* is a FERM domain containing protein. FERM (4.1/Ezrin/Radixin/Moesin) domain containing proteins are known to link the actin cytoskeleton the membrane of the cell by binding to transmembrane proteins. In an ENU-induced allele of *Epb4.1l5* (*lulu*), the cells accumulate at the streak in an intermediate step of EMT as they still retain epithelial characteristics (Lee et al., 2007). Additionally, Ebp4.1l5 post-transcriptionally regulates the decrease in E-cadherin levels and increase in integrin based signaling at the streak. This is mediated by its interaction with p120-catenin and Paxillin (Hirano et al., 2008).

1.3 Signaling pathways at the primitive streak

In addition to being the site of EMT, the primitive streak is a hub of signaling pathways. Mutations in genes that directly or indirectly affect these signaling pathways alter the process of gastrulation. In addition, signaling pathways at the streak regulate the expression of EMT transcription factors. Therefore, in the

following section I will review the signaling pathways that are involved in the establishment and maintenance of the mammalian primitive streak.

1.3.1 Initiation of the primitive streak

The TGF-β family member Nodal and Wnt signaling establish the proximal-distal axis of the embryo. They specify the DVE (distal visceral endoderm) cells and induce expression of secreted antagonists of Nodal and Wnt signaling (Lefty1, Cerberus like and Dickkopf-related protein 1) (Perea-Gomez et al., 2002). The DVE cells actively migrate towards the embryonic-extra-embryonic border and become the Anterior Visceral Endoderm (AVE) (Srinivas et al., 2004). This breaks the symmetry of the embryo as the presence of the inhibitors on the anterior side results in a gradient of Nodal and Wnt signaling. Despite the signaling gradients, the embryo is still morphologically radially symmetrical. The formation of the primitive streak on the posterior side of the embryo breaks the radial symmetry and establishes the anterior-posterior axis of the embryo.

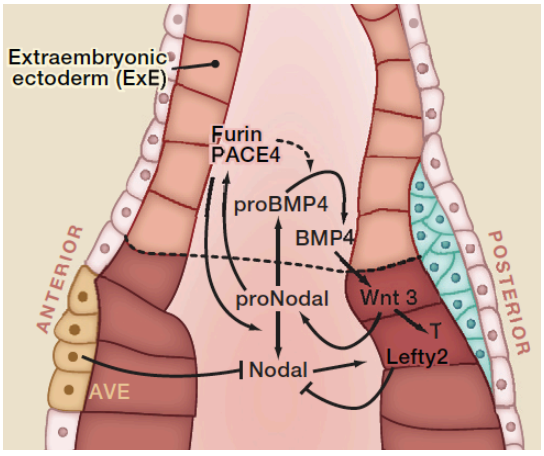


Figure 1.6 Establishment of primitive streak
Signaling by *Nodal*, *BMP4* and *Wnt3* help establish the streak at the posterior side of the embryo. The AVE cells restrict the signaling to the posterior side.

The establishment of the primitive streak involves extensive signaling across the embryo. *Nodal*, *BMP4* and *Wnt3* are responsible for the initiation of the primitive streak. Mice lacking *Nodal* or *Wnt3* never form a primitive streak or mesoderm (Conlon et al., 1994; Liu et al., 1999). A small percentage of mice homozygous for null allele of *BMP4* form a primitive streak, but die shortly after due to failure of maintenance of the streak (Winnier et al., 1995). *Nodal* induces two auto feedback loops maintaining its levels and also induces other pathways in this process. Nodal is derived from its secreted precursor proNodal, which is processed by secreted proteases of Subtilisin-like proprotein convertase (SPC family) Furin and PACE4. proNodal in the epiblast induces the expression of Furin and PACE4 in the extra-embryonic ectoderm (ExE). Once processed, mature Nodal auto-regulates its own expression. Simultaneously, proNodal induces the expression of *BMP4* in the ExE and this induces the expression of *Wnt3* in the posterior epiblast cells (Ben-Haim et al., 2006). *Wnt3* activates expression of Brachyury, a hallmark of the primitive streak (Rivera-Perez and Magnuson, 2005). Lefty-1, Cerberus-like and Dkk1 expressed in the AVE cells restrict Nodal and Wnt signaling respectively to the posterior side and therefore endow neuroectoderm fate to the anterior epiblast (Kimura-Yoshida et al., 2005; Perea-Gomez et al., 2002). Wnt3 positively maintains the expression of Nodal via the proximal epiblast enhancer of Nodal, initiating the second feedback loop (Ben-Haim et al., 2006). Nodal levels on the posterior epiblast are negatively regulated by its target Lefty2 (Meno et al., 1999).

1.3.2. Maintenance of the primitive streak

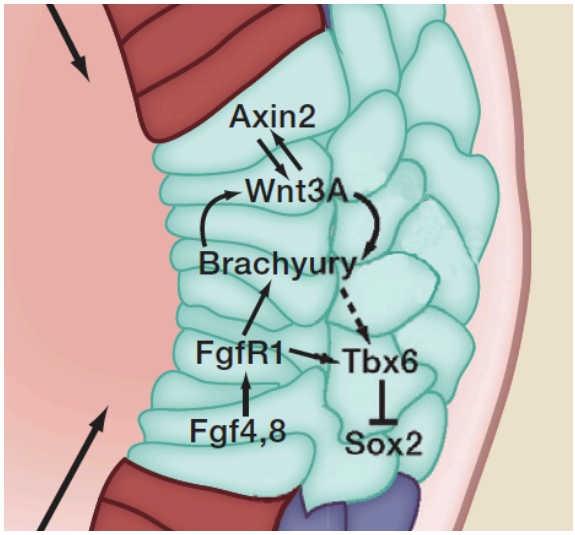


Figure 1.7 Maintenance of the primitive streak
Signaling loops initiated by *Wnt3A*, *Brachyury* and *Fgfs* maintain the primitive streak on the posterior side of the embryo.

At the onset of gastrulation, *Wnt3* is expressed in the primitive streak. As gastrulation proceeds, *Wnt3A* replaces *Wnt3* at the primitive streak and turns on the expression of T-box family of transcription factors Brachyury (T) and Tbx6 in the streak (Yamaguchi et al., 1999). These in turn maintain the expression of *Wnt3A* and establish a robust feedback loop. *Wnt3A* signaling is also regulated by *Axin2*, a direct target of Wnt signaling and a member of Wnt destruction complex (Qian et al., 2011). *Tbx6* acts as a switch between mesoderm and neural lineage as it negatively regulates *Sox2* expression, therefore inhibiting neural fate in the cells where it is expressed (Takemoto et al., 2011). Thus expression of *Wnt3A* and *Tbx6* is essential for progenitors to adopt a mesodermal fate over neural fate. However, they are not essential for cell ingression as the cells can ingress in

Wnt3A and *Tbx6* mutants but they give rise to ectopic neural tubes at the expense of paraxial mesoderm (Yoshikawa et al., 1997).

FGF signaling reinforces the expression of T-box transcription factors *Brachyury* and *Tbx6* and therefore maintains the primitive streak. *FGF4* and *FGF8* are transiently expressed in the streak and activate FGFR1, which maintains the expression of *Brachyury* and *Tbx6* (Ciruna and Rossant, 2001).

1.4 Summary:

The primitive streak is a hub of signaling pathways and also a center for organized EMT. While mutations in any component of the signaling hub directly effect the establishment and maintenance of the streak, a second class of mutants specifically affect the process of gastrulation EMT. Analysis of these mouse mutants has contributed extensively to our knowledge on the various aspects and regulators of gastrulation EMT. However, there are still many open questions and interesting observations that demand a further exploration of this process. For instance, the fact that cells can ingress without down-regulation of *E-cadherin* in *Snail1* mutants suggests that down-regulation of *E-cadherin* is not essential for cell ingression. This warrants a re-examination of other gastrulation mutants wherein the defect was attributed to *E-cadherin* down-regulation.

The mechanical properties that define EMT have not been investigated. The primitive streak is a few cells wide and runs to the distal tip of the embryo. Questions such as how many cells delaminate at a given time, factors that dictate which cells can delaminate, and how is the integrity of the epiblast maintained while cells constantly delaminate remains unresolved. Additionally, it remains to

be determined if cell ingression by itself is an active process or if cells just passively move towards the streak. The relevance of apical constriction or cell shape changes at streak also needs to be determined.

1.5. Glycosylation of Proteins

Protein glycosylation is a common post-translational modification that involves the covalent addition of sugar moieties to the protein backbone. Glycosylation of proteins has been observed in all species from bacteria to humans and is one of the most abundant post-translational modifications, as more than fifty percent of all proteins can be glycosylated (Apweiler et al., 1999). Following the initial covalent linkage, these sugar moieties can be extended to form long carbohydrate chains. The numerous permutations and combinations of the sugars in the carbohydrate chain and their possible branching patterns make glycosylation one of the most structurally diverse PTMs (Cummings, 2009).

Glycosylation of proteins has been shown to regulate their stability and function by being a quality control and ensuring efficient folding of proteins and altering their affinity to interacting partners and ligands (Bruckner et al., 2000; Imperiali and Rickert, 1995; Moremen and Molinari, 2006; Okajima et al., 2008; Okajima et al., 2003; Panin et al., 1997). Altered glycosylation of proteins has been observed in cancer, inflammatory and neuro-degenerative diseases and thought to promote the disease progression (Bull et al., 2014). The importance of glycosylation is apparent from the large number of human diseases caused by congenital disorders of glycosylation (CDGs), such as congenital muscular dystrophies, Peters'-plus syndrome and many others, which are categorized based on the sugar linkage and enzymes mutated in human patients (Freeze,

2013; Freeze and Aebi, 2005; Schachter and Freeze, 2009). Additionally, developmental defects in mice with mutations in glycosyltransferases suggests the importance of protein glycosylation in mammalian development (Du et al., 2010; Fernandez-Valdivia et al., 2011; Garcia-Garcia and Anderson, 2003; Okamura and Saga, 2008; Willer et al., 2004). Due to the broad spectrum of glycan structures in the glycome, much less is known about glycosylation relative to other PTMs like phosphorylation.

Glycans are classified based on the nature of their linkage to the protein backbone. If the carbohydrate is covalently linked to the amide nitrogen of Asparagine it is called N-linked while if the linkage is to the oxygen of the hydroxyl group of Serine/Threonine or hydroxyl lysine it is called O-linked. Another class of linkage where the carbohydrate and protein are linked by a carbon-carbon bond, is called C-linked. One of the mouse mutants described in this thesis *wsnp* is an allele of Protein O-glucosyltransferase 1, which affects O-glucosylation of proteins. Therefore, I will focus on this sugar modification and its importance in mammals.

1.5.1. O-glycosylation of proteins

O-glycosylation is a sugar modification that is characteristic of protein domains called Epidermal Growth Factor (EGF) repeats. EGF repeats are small motifs containing six conserved cysteines, which form three disulfide bridges. EGF repeats are known to contain two kinds of O-linked glycosylation, O-glucose and O-fucose, wherein the respective sugar moieties are covalently linked to the Serine/Threonine within their consensus sequence on EGF repeats (Harris and

Spellman, 1993). The corresponding glycosyltransferases are expressed in the endoplasmic reticulum, where these sugars are added to the protein backbone.

Initial studies identified the presence of these sugar modifications on EGF repeats of proteins (Harris and Spellman, 1993). Extensive biochemical studies led to the purification of the corresponding enzymes from cell extracts. Eventually, the genes coding for these enzymes were cloned and their biological functions analyzed.

Protein O-fucosyltransferase1 is the enzyme that adds O-fucose to mammalian EGF repeats (Wang et al., 2001). The function of O-fucosylation has been extensively investigated. Since its identification, it has been found on the EGF repeats of many cell surface proteins. Fucose can be further extended by other glycosyltransferases such as Fringe. These sugar modifications alter the affinity of the ligands to the receptor (Panin et al., 1997). In addition to its enzymatic activity, Pofut1 was also shown to function as a chaperone in the ER (Okajima et al., 2008).

1.5.2. O-glucosylation of EGF repeats

Following the cloning of Pofut1, an enzymatic activity responsible for the addition of O-glucose to EGF repeats was identified (Shao et al., 2002). This activity was present in protein extracts in a number of species from *Drosophila* to humans. However, no protein was identified with this enzymatic activity until the identification of *Rumi*. *Drosophila Rumi* was identified in a screen for regulators of Notch signaling. It was shown to have enzymatic activity of adding O-glucose to EGF repeats (Acar et al., 2008). The mammalian homologue Protein O-

glucosyltransferase1 (Poglut1; also called Ktelc1) was identified and characterized to have O-glucosylation activity towards EGF repeats (Fernandez-Valdivia et al., 2011). *Rumi* is important for Notch signaling in *Drosophila*. Studies in mammalian cell lines also suggest a conserved role for *Poglut1* in Notch signaling in mammals (Acar et al., 2008; Fernandez-Valdivia et al., 2011). The biologically relevant targets and functional significance of these modifications on proteins during mammalian development remain unknown.

With the help of a mouse mutant *wsnp*, an allele of *Poglut1*, I show that the function of Poglut1 in Notch signaling is conserved in mammals. Additionally, I identify mammalian Crumbs2 as an important biologically relevant target of Poglut1 during mammalian gastrulation. In the following sections, I will review the functions of Notch and Crumbs protein during gastrulation and the significance of their O-glucosylation during this process.

1.6. Notch signaling

Notch is a cell surface receptor identified in *Drosophila* because dominant mutations in the gene cause notches in the fly wing. Together with its ligands, it plays an important role in cell-fate decisions at various stages of development. The classic loss of Notch function phenotype in *Drosophila* is neurogenic wherein cells fail to segregate into neural and ectoderm lineages. Since its discovery, extensive studies have identified the core components of canonical Notch signaling, which are highly conserved across species. The protein has a very large extracellular domain (1693 amino acids) containing 36 Epidermal Growth Factor repeat domains, a heterodimer domain (HD), and three LNR (Lin-12, Notch repeats) domains, followed by transmembrane domain, ankyrin repeats, and a PEST motif. The domain organization of Notch is conserved across species.

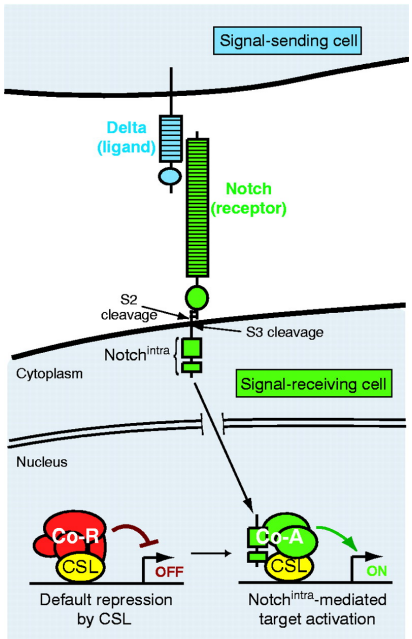


Figure 1.8 Basic Notch signaling pathway.
Series of proteolytic cleavage release the intracellular domain of Notch, which goes to the nucleus and binds transcription complex to turn on expression of target genes. Adapted from (Lai, 2004).

Notch is expressed on the cell surface as a hetero- dimer as it undergoes the first proteolytic cleavage (S1) mediated by furin in the Golgi before it is expressed on the cell surface (Logeat et al., 1998). The significance of this proteolytic cleavage remains debated. The canonical pathway involves a series of proteolytic cleavages. Once on the cell surface, Notch binds to its ligands, which is thought to introduce a conformation change that facilitates its cleavage (S2) by the ADAM10 metalloprotease. This is followed by an intramembranous S3/4 cleavage mediated by the Presenilin family protein γ-secretase, which releases the Notch intracellular domain (NICD) from the membrane. NICD goes into the nucleus and binds to transcription co-factor *Rbpjk* (Suppressor of Hairless) to activate transcription of downstream target genes (Tien et al., 2009). In mammals there are four Notch paralogues (*Notch1-4*) and five ligands (*Delta like 1,3,4 and Jag1 and Jag2*). Together they activate the expression of *Hes* and *Hey* transcription factors and other targets.

Unlike most signaling pathways, Notch signaling does not have any secondary messengers, which would amplify the signal. Therefore cells are highly sensitive to the levels and genetic dosage of Notch signaling. As a result, both activating and loss of function mutations in the Notch pathway are deleterious and cause inherited syndromes and cancer (Gridley, 2003; Penton et al., 2012). Mutations in Notch signaling can be oncogenic or tumor suppressive depending on the context (South et al., 2012). The Notch pathway is tightly regulated to control the levels of signaling. It is regulated by glycosylation of its extracellular domain, intracellular trafficking and ubiquitination of both the ligand and the receptor (Schweisguth, 2004).

1.6.1 Notch signaling in mammalian gastrulation

Notch signaling has many important roles during mouse development owing to its central function in cell fate decisions. The earliest established role for Notch signaling in mouse development is somitogenesis. Mutations in Notch pathway have been associated with altered somitogenesis. Inherited human diseases with skeletal abnormalities like spondylocostal dysostosis (SCDO) are caused due to mutations in Notch signaling (Shifley and Cole, 2007). Paraxial mesoderm derived from the primitive streak undergoes repeated segmentation to give structures called somites, which are present on either side of the neural tube. The somites are transient embryonic structures, which will eventually give rise to the bones and musculature in the adult. This segmented body plan has been observed in all vertebrates.

The presomitic mesoderm (PSM) comprises a subset of the cells that have ingressed through the primitive streak. In mice this process is initiated at E7.75-E8.0. Somite formation occurs sequentially with addition of a new somite pair to the anterior side of the PSM. The segmentation clock regulates the periodic generation of somites (Hubaud and Pourquie, 2014). Various signaling pathways are activated in a cyclic manner in the PSM, which ensures proper segmentation of the PSM into somites.

Notch and its target genes are expressed in oscillatory manner owing to the feedback loops (Ferjentsik et al., 2009). Delta-mediated Notch signaling turns on the expression of *Hes7* and *Lunatic Fringe* in the PSM. *Hes7* and *Lunatic Fringe* down regulate Notch signaling leading to oscillatory expression of these genes.

The earliest expression of *Notch* and its ligands have been described in the mouse embryo at E7.0. In situ hybridization for *Notch1* shows that it is in strongly expressed in the nascent mesoderm (Reaume et al., 1992). Immunostaining for activated Notch1 with an antibody specific to cleaved Notch1 shows that at this stage the pathway activation is restricted to nascent mesoderm (Del Monte et al., 2007). Among the Notch ligands, *Delta like1 and 3* are also expressed in the primitive streak and nascent mesoderm at this stage (Bettenhausen et al., 1995; Dunwoodie et al., 1997). Since somite formation is visible at E7.75, it would be interesting to speculate that this process is initiated even before that. Recent evidence with a more sensitive fluorescent reporter for Notch activity suggests that the pathway is active in the epiblast of early post implantation embryos even before the commencement of gastrulation (Nowotschin et al., 2013). The functional role of Notch pathway at this stage remains to be determined.

1.6.2. Redundancy of Notch function in mammalian development

Unlike *Drosophila*, which has only one *Notch*, the redundancy of the pathway in mice complicated its analysis. Mammals have four Notch receptors and five ligands. Among the ligands and receptors, targeted disruption of *Notch1, Notch2, Dll1, Jagged1 and Jagged2* lead to embryonic lethality. Mice lacking *Notch1* develop normally until mid gestation and die around E11.5 due cardiovascular abnormalities. As expected, they have delayed and disorganized somites (Conlon et al., 1995). Mice lacking the ligand *Delta like 1* also die around the same time with similar irregular somites (Hrabe de Angelis et al., 1997). To date, no one has analyzed mouse mutants lacking all four Notch receptors or all ligands. However, mice lacking *ADAM10* metalloprotease and γ-secretase, which are essential for generating cleaved/active Notch, have been generated. Mice lacking these genes

have a more severe phenotype compared to just loss of *Notch1*. Mice lacking *ADAM10* die around E9.5 due to multiple defects in neurogenesis, somite formation and cardiovascular abnormalities (Hartmann et al., 2002). Mice lacking both *Presenilins* (*Presenilin 1 and 2*) die around E9.5 due to defects in trunk neural tube, absence of somite segmentation and abnormal heart. Analysis of downstream targets of the Notch pathway reveals that Notch signaling is down regulated in these mutants (Donoviel et al., 1999). A major caveat with these mutants is that while Notch signaling is perturbed in these mutants, we cannot rule out the involvement of other pathways in the phenotypes of these mice. *Presenilins* have been shown to interact with many other proteins like β-catenin and Rab11 (Parks and Curtis, 2007). Therefore, the mutants could represent additional roles of *Presenilin* in mouse development.

Mammals have a single transcription factor *RbpjK* that interacts with NICD to activate target genes. Mice lacking both embryonic and zygotic *RbpjK* die around E10.5. They have defects in neural tube closure, somitogenesis and pericardial edema (Souilhol et al., 2006). The defects in these mice were more severe than loss of *Notch1*. Studies in *Drosophila* show that *Suppressor of Hairless* (*RbpjK* homologue) can function both as an activator and a transcriptional repressor. Additionally, not all targets of Notch signaling were down regulated in *RbpjK* mutants and the CSL complex has been shown to affect the transcription of genes independent of Notch pathway (de la Pompa et al., 1997; Johnson and Macdonald, 2011). Due to the lack of clean null for Notch pathway, its roles in early embryogenesis remains to be completely determined.

1.6.3. Role of glycosylation in Notch signaling

The Notch protein has multiple EGF repeats on its extracellular domain. The extracellular domain of Notch is extensively glycosylated by two important class of O-linked modification namely O-fucose and O-glucose, which are essential for its function.

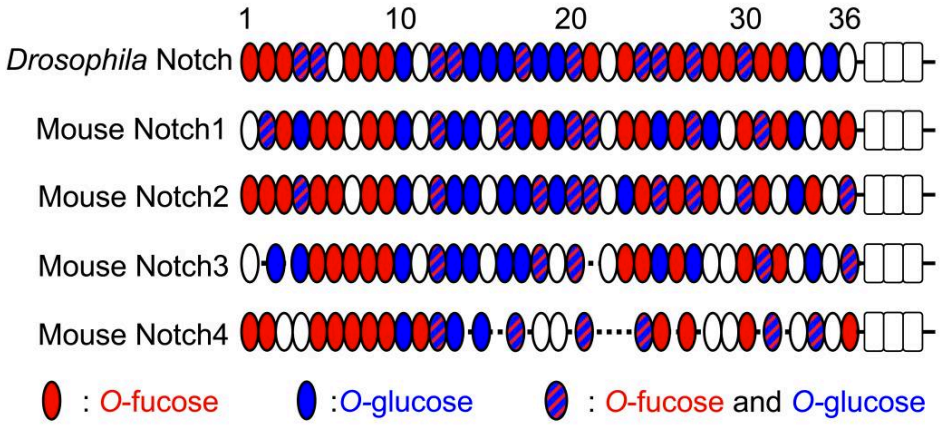


Figure 1.9 O-Glycosylation of extracellular domain of Notch
Alignment of EGF repeats on the extracellular domain of Drosophila and Mammalian Notch showing potential O-fucosylation and O-glucosylation sites. Adapted from (Takeuchi and Haltiwanger, 2010)

O-fucose is added in the ER and further extended to GlcNAc β 1-3Fuc disaccharide by the action of *N*-acetylglucosaminyltransferase Fringe in the Golgi (Jafar-Nejad et al., 2010). In mammals there are three homologs of *Fringe* namely *Lunatic Fringe, Radical Fringe* and *Manic Fringe*. Fringe activity on Notch EGF repeats modulates its binding to its ligands. Protein O-fucosyltransferase1 is the only enzyme known to have the O-fucosylation activity towards EGF repeats. Mouse mutants lacking *Pofut1* have defects in cardiogenesis, neurogenesis and somitogenesis similar to *RbpjK* and *Presenilin* mutants (Okamura and Saga, 2008). Although the presence of O-fucose on EGF repeats has been shown only

for Notch1, the other mammalian Notch proteins are predicted to have this modification.

Drosophila Rumi was identified in a genetic screen because of a Notch-like mutant phenotype. Rumi was shown to modify the EGF repeats on the extracellular domain of Notch receptor. *Rumi* mutants had a temperature dependent loss of Notch signaling. At high temperatures, Notch receptor could not be cleaved upon binding to its ligand and therefore led to a loss of Notch phenotype in *Rumi* mutants. O-glucosylation of the extracellular domain of Notch was essential for proper folding of Notch in order to facilitate its S2 cleavage by metalloproteases upon ligand binding (Acar et al., 2008). Unlike Pofut1, which also functions as a chaperone, Poglut1 functions primarily as an enzyme, as expression of protein with mutated enzymatic domain could not rescue the *Rumi* phenotype (Acar et al., 2008; Okajima et al., 2005). In contrast to sugar modifications by *Fringe*, the ligand-receptor affinity was not altered in the unmodified Notch (Acar et al., 2008). Among the mammalian homologs, Poglut1 was the only protein shown to have enzymatic activity towards EGF repeats. Like Pofut1, it has an ER retention signal (KTEL) in its C-terminal. Poglut1 was shown to modify Notch in mammalian cell lines and this modification was shown to affect Notch signaling at a step between ligand binding and S3 cleavage of Notch (Fernandez-Valdivia et al., 2011).

The O-glucose is further extended by two α 1-3 linked xylose residues. This trisaccharide has been observed in all EGF repeats that contain an O-glucose modification. Recent evidence in *Drosophila* suggests that the addition of this xylose to extend the O-glucose could inhibit trafficking of Notch, suggesting this

could be similar to Fringe modulating Notch activity (Lee et al., 2013). Understanding the functional role of O-glucose modification requires further investigation.

Mice lacking *Poglut1* have a phenotype more severe than known mutants of the Notch pathway including *RbpjK* and *Presenilins* and the effect of this sugar modification on the Notch pathway in the embryo has not been investigated. Additional studies with Poglut1 were conducted in cell lines, which showed an effect on Notch signaling. In this thesis I will describe the functions of Poglut1 in mouse embryonic development.

1.7 Crumbs

Epithelial cells have a well-defined apical-basal axis whose maintenance is essential for homeostasis. The formation and maintenance of this axis requires many factors such as scaffolding proteins and their interacting partners expressed in different domains of the cell, polarized trafficking and cytoskeletal organization to facilitate this process. The scaffolding proteins help in establishing this axis and based on their interactions and feedback loops, maintain this organization. Three major protein complexes have been identified as polarity regulators and they are conserved from invertebrates to vertebrates. The Crumbs complex and the Par complex are apical polarity regulators, while Scribble is basolateral. The members of these complexes interact extensively to maintain the epithelial polarity. In my thesis, I will be describing the potential roles of Crumbs proteins in mammalian gastrulation with the help of mouse mutants. In the following section I will review the known functions of Crumbs proteins in epithelial polarity and signaling.

Crumbs was the first apical polarity determinant identified in *Drosophila.* Loss of Crumbs protein leads to a disruption of polarity in the embryonic ectoderm and eventually to its degeneration. *Crumbs* was originally identified in a genetic screen for mutants with defects in cuticle. Since the mutants have a discontinuous cuticle with holes it was named Crumbs. The gene responsible for this phenotype was identified as an apically expressed protein with multiple EGF repeats required to maintain the embryonic epithelia (Tepass et al., 1990).

Among the polarity regulators, Crumbs is the only transmembrane protein and is highly conserved from *C. elegans* to humans. While *Drosophila* has only one *Crumbs* gene, mammals have three members, namely *Crumbs1, Crumbs2* and *Crumbs3*. Extensive work has been done on the many roles of Crumbs in cell polarity, tissue morphogenesis particularly photoreceptor morphogenesis and its role in signaling pathways such as Hippo and Notch. I will discuss some of the work done in various systems to understand the mechanism of Crumbs function.

1.7.1. Canonical role of Crumbs

In addition to its apical expression, early experiments in *Drosophila* established its role as an apical determinant. Overexpression of full-length protein caused an expansion of the apical domain and a subsequent reduction in the basolateral domain of cells in the epithelia. Crumbs is essential for the maturation of adherens junctions in the embryonic ectoderm. In absence of Crumbs, the junctions fail to form properly, and this defect precedes the abnormal cell shape and tissue architecture that eventually leads to breakdown of the epithelia (Tepass, 1996). The eventual disorganization of the epithelia in the absence of Crumbs has been observed in all tissues wherein its role has been investigated

including the zebrafish retina and neural epithelium (Malicki and Driever, 1999; Omori and Malicki, 2006). *Drosophila* Crumbs has also been associated with regulating cell shape in the embryonic ectoderm and salivary placode. Cells expressing high levels of Crumbs have a more constricted wedge shaped morphology (Letizia et al., 2013).

Crumbs has a very large extracellular domain (1994 amino acids), whose function is largely unknown, and a short cytoplasmic tail of only 37 amino acids. The intracellular domain of Crumbs was established as the functional domain of the protein since its expression could confer the apical characteristics similar to the full-length protein. Additionally, expression of this domain could moderately rescue some aspects of the cuticle phenotype of *Crumbs* mutant (Wodarz et al., 1995).

The short cytoplasmic tail of Crumbs contains two highly conserved domains, a PDZ (PSD-95/Discs large/ZO-1)-binding, carboxy-terminal ERLI motif and a membrane proximal FERM (4.1/ezrin/radixin/moesin)-binding domain. The PDZ domain is required to recruit the canonical Crumbs complex consisting of Stardust (Sdt), DPatJ and DLin7. It can also interact with aPKC and Par6. The core Crumbs complex functions as a unit to stabilize the membrane expression of Crumbs. Absence of any one of the components leads to a failure of apical expression of Crumbs and therefore a phenotype similar to the loss of Crumbs function (Knust et al., 1993; Penalva and Mirouse, 2012; Zhou and Hong, 2012). The core Crumbs complex is also conserved across species. Recently it was shown that the complex proteins might have additional functions independent of Crumbs (Zhou and Hong, 2012).

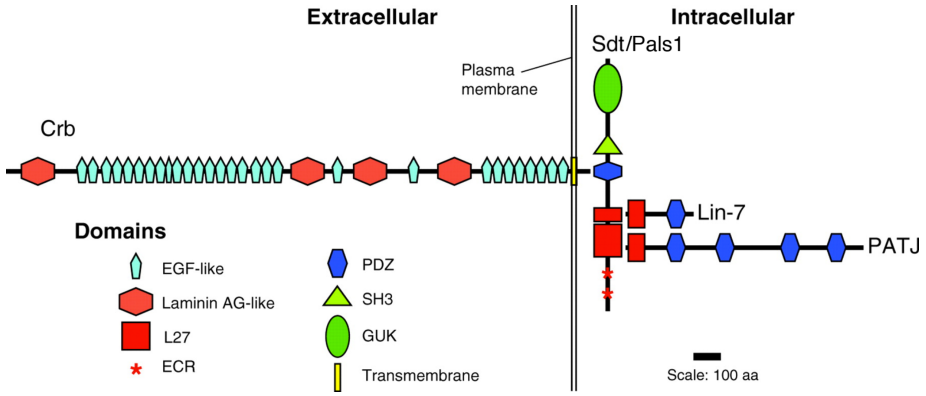


Figure 1.10 The core Crumbs complex (Bulgakova and Knust, 2009)
The complex consists of Pals1, PatJ and Lin-7. This complex is conserved from *Drosophila* to humans.

The FERM-binding domain is important for the recruitment of cytoskeletal regulators. Among the FERM domain proteins, Moesin, Yurt and Expanded were shown to physically interact with Crumbs. Yurt (mammalian Epb4.1l5) negatively regulates the domain of Crumbs expression and restricts it to the apical membrane. *Yurt* mutants have an expansion of the Crb domain similar to the over-expression of Crumbs (Laprise et al., 2006; Laprise et al., 2009). Moesin was shown to regulate the apical membrane cytoskeleton by mediating the interaction of Crumbs and beta-heavy spectrin. Besides coimmunoprecipitating as a complex, clustering of a chimeric intracellular domain of Crumbs led to recruitment of both Moesin and beta-heavy spectrin. Together, this complex maintains the apical membrane skeleton (Medina et al., 2002). The FERM-binding domain of Crumbs also interacts with Expanded (another FERM domain protein) and thereby links Crumbs to the Hippo signaling pathway. Crumbs not only recruits Expanded to the apical membrane but also promotes its degradation by

ubiquitin ligases and therefore activates *Drosophila* Yorkie (Chen et al., 2010; Ling et al., 2010; Ribeiro et al., 2014; Robinson et al., 2010).

Besides these three, other potential FERM proteins interacting with Crumbs remain to be determined. The FERM-binding domain of Crumbs is dispensable for cell polarity but is essential for dynamic events such as dorsal closure and germ band retraction (Klose et al., 2013).

The levels of Crumbs are tightly regulated as both overexpression and loss of Crumbs leads to a defect in epithelial integrity. The trafficking machinery plays an essential role in regulating Crumbs level. Retromer regulates the retrograde recycling of Crumbs. Retromer is essential for the retrograde transport of numerous transmembrane proteins from endosomes to the *trans*-Golgi network (TGN). Loss of retromer functions leads to reduction in the amount of Crumbs and therefore phenocopies the defects observed in *Crumbs* mutants (Pocha et al., 2011).

1.7.2. Mammalian Crumbs

In mammals, there are three members of the Crumbs family (*Crb1, Crb2 and Crb3*) with highly similar intracellular domains. Crumbs1 and Crumbs2 have very large extracellular domains similar to *Drosophila* Crumbs. Crb3 has a very short ECD with no similarity to any Crumbs family member and has two splice isoforms *Crb3a* and *Crb3b*, which differ in their C-terminal sequences. The core components of the Crumbs complex (PatJ, Pals1 and Lin-7) are conserved in mammals.

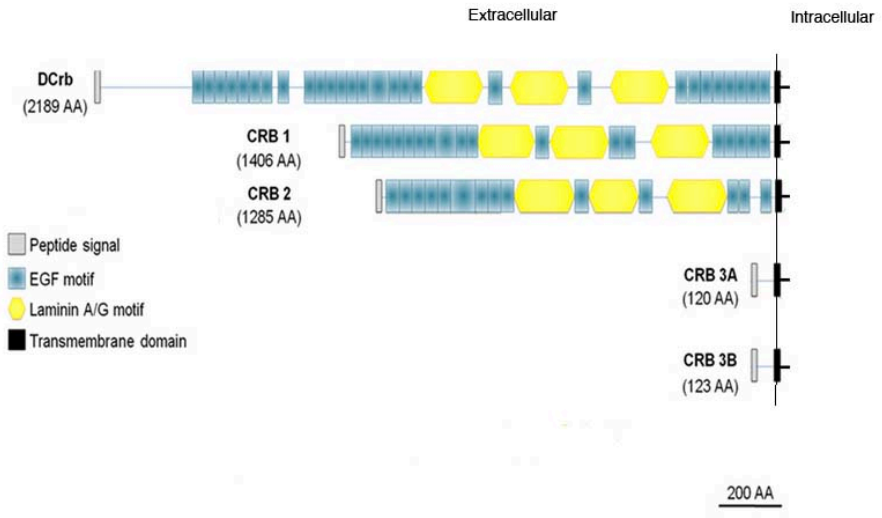


Figure 1.11 Domain organization of mammalian Crumbs proteins.
Crb1 and Crb2 have huge extracellular domains while Crb3 have a short one. They all have a highly conserved intracellular domain.

Crumbs1 was the first member of the family to be cloned. Mutations in human *Crumbs1* are associated with severe retinal dystrophies like Leber congenital amarousis and Retinitis pigmentosa (den Hollander et al., 2004; den Hollander et al., 1999). *Crumbs1* is expressed specifically in the eye and the central nervous system (den Hollander et al., 2002). Mice homozygous for a spontaneous mutation allele of *Crumbs1 (rd8)* have photoreceptor dysplasia and degeneration. Similarly, the retina of mice homozygous for the null allele, *Crumbs1*-/-, undergoes light dependent degeneration, despite normal development (van de Pavert et al., 2004). Functionally, expression of the intracellular domain of human Crumbs1 could rescue cuticle defects in *Drosophila Crumbs* mutants (den Hollander et al., 2001).

Because of its small size and wide expression, *Crumbs3* has been extensively studied in cell lines. Over expression and knockdown of *Crumbs3* affected the dynamics of tight junction formation in MDCK cells (Fogg et al., 2005; Roh et al., 2003). Additionally, a novel isoform of *Crumbs3* (*Crb3b*) was shown to regulate formation of primary cilia (Fan et al., 2007). The transcription factor *Snail1* can regulate the levels of Crumbs3 and thereby promote EMT in MDCK cells (Harder et al., 2012; Whiteman et al., 2008). Crumbs3 functions as a tumor suppressor in epithelial tumors (Karp et al., 2008). Mice homozygous for a null allele of *Crumbs3* die at birth due to defects in lungs, kidney and intestinal epithelium. Despite its strong role in tight junction formation in MDCK cells, tight junctions form normally and remain unaffected in *Crumbs3* mutant mice (Whiteman et al., 2014). Based on recent findings, the function of *Crumbs3* in epithelia is still unclear and is thought to affect apical membrane processes like microvilli.

Mammalian *Crumbs2* is probably the least studied member of the family. Its expression is very limited in the adult and until recently there wasn't any association to human diseases. Mutations in human *Crumbs2* were recently associated with congenital nephrosis and steroid resistant nephrotic syndrome (Ebarasi et al., 2015; Slavotinek et al., 2015). Additionally, loss of *Crumbs2* in the mouse retina also causes Retinitis pigmentosa (Alves et al., 2013; Pellissier et al., 2014). Mice homozygous for a null allele of *Crumbs2* are embryonic lethal at E9 (Xiao et al., 2011). They have major defects in mesoderm-derived tissues, indicating a late gastrulation phenotype. The phenotype was attributed to defects in epiblast polarity at the primitive streak leading to disrupted gastrulation EMT. However, that study does not describe any defects in the localization of apical components, suggestive of defects in cell polarity. The only defect described is

abnormal basement membrane integrity, which is a sign of epithelial degeneration, a phenomenon observed in most tissues with loss of Crumbs function (Xiao et al., 2011). Therefore the function of *Crumbs2* in gastrulation still remains to be determined.

1.7.3. Functions of the extracellular domain of Crumbs

The extracellular domain of *Drosophila* Crumbs has 29 EGF like repeats and four Laminin A like globular domains. Crumbs1 and Crumbs2 have 19 and 15 EGF like repeats respectively and two laminin A/G domains. Mutations in *Crumbs1* and *Crumbs2* associated with human diseases predominantly map to the extracellular domain of Crumbs, suggesting an important function of this domain (den Hollander et al., 2004).

While most of the work with Crumbs focused on its conserved intracellular domain, the importance of the extracellular domain came into focus with the role of Crumbs in photoreceptor morphogenesis. *Drosophila* Crumbs is expressed in a specialized apical compartment called the stalk of photoreceptor cells (PRC). Crumbs regulates the length of the stalk domain in PRCs thereby controlling the physical distance between the rhabdomeres and preventing their collapse. Genetic mosaic analysis in the *Drosophila* retina showed that loss of Crumbs leads to cells with a shorter stalk region and eventual collapse of the rhabdomeres. This function is specific to the membrane bound extracellular domain of Crumbs, as its expression could rescue the stalk length defect in the mutants (Izaddoost et al., 2002; Pellikka et al., 2002). Besides regulating stalk length, Crumbs is required to prevent light induced retinal degeneration in the PRCs. The ECD of Crumbs mediates this function as expression of ECD can rescue the light induced

degeneration (Johnson et al., 2002). Additionally, the ECD of *Drosophila* Crumbs is important to stabilize its membrane localization (Letizia et al., 2013), probably by homophilic interactions in *trans*.

Analysis of *Drosophila* mosaic clones with wild type and *Crumbs* mutant cells suggested a potential homophilic interaction between the Crumbs ECD in *trans*. The edges shared between a wild type cell and mutant cells always lacked Crumbs protein expression. Evidence for the functional importance of these interactions comes from studies in the zebrafish retina. Zebrafish have five homologs in the Crumbs family (*Crb1, Crb2a, Crb2b, Crb3a and Crb3b*). They are differentially expressed during development and are thought to have partially overlapping functions. Among them, *Crb2a* plays a major role in retinal and neural tube morphogenesis (Malicki and Driever, 1999). Homophilic and heterophilic interactions between the extracellular domains of Crb2a and Crb2b are essential for maintaining cone mosaics in zebrafish retina (Zou et al., 2012).

Besides homophilic interactions, other ligands or interacting proteins have not yet been identified. Much work remains to be done to investigate the role of the ECD in Crumbs functions. In this thesis I will show data that suggest that the EGF repeats on the ECD of Crumbs2 are glycosylated by Poglut1. This O-glucosylation is essential for its apical membrane localization, which in turn is essential for its function.

1.7.4. Crumbs and morphogenesis:

Crumbs is expressed in most but not all epithelia in *Drosophila*. Its expression is first turned on during gastrulation. Following gastrulation and germ band

extension, it is expressed in the ectodermal primordia of foregut, the tracheal system, the salivary glands, the malpighian tubules, the optic lobes, and the stomatogastric nervous system (Tepass et al., 1990). A feature common to these tissues is that their specification requires tissue invagination. A considerable body of work has demonstrated the requirement of Crumbs in malpighian tubules, tracheal placode, photoreceptor morphogenesis and salivary gland morphogenesis (Campbell et al., 2009; Letizia et al., 2011; Pellikka et al., 2002; Pirraglia and Myat, 2010). In most of these tissues, Crumbs function is essential during tissues morphogenesis, with enhanced expression in invaginating tissues.

Morphogenesis involves dynamic rearrangements of cell junctions, which requires constant turnover of adherens junctions and cytoskeletal reorganization. If these cell rearrangements are blocked, the defects in cell polarity in *Crumbs* mutants can be partially rescued (Campbell et al., 2009). This suggests that tissues undergoing rearrangement need an additional mechanism to reinforce epithelial integrity.

The requirement of Crumbs in morphogenetic events suggests that it regulates an aspect of cell rearrangement. It could directly regulate the dynamics of adherens junctions. Since Crumbs does not co-localize with E-cadherin or physically interact with E-cadherin, the nature of their relationship remains unclear. Alternatively, Crumbs could regulate cytoskeletal reorganization to facilitate cell rearrangements.

A potential role for Crumbs in cytoskeletal reorganization was established in organization of actomyosin cable in salivary placode during *Drosophila* salivary gland morphogenesis (Roper, 2012). Myosin cable formation is essential for

invagination during salivary gland morphogenesis. A detailed characterization of Crumbs expression in the salivary placode shows an anisotropic pattern with high anti-correlation between Crumbs localization and the site of myosin cable formation. Cells expressing high Crumbs recruit aPKC, which inactivates Rho-Kinase, a kinase that regulates myosin activity and therefore prevents cortical myosin accumulation. In contrast cells expressing low levels of Crumbs form stable myosin cables. Similar phenomena have yet to be documented in other tissues during invagination.

1.7.5. Summary:

Crumbs is the only transmembrane protein that regulates epithelial integrity in tissues undergoing dynamic rearrangements. Despite extensive investigation on the functions of Crumbs since its identification 25 years ago, the mechanism of its action still remains unclear. It is speculated to regulate E-cadherin dynamics. However, as mentioned before, there is no over-lap between Crumbs and E-cadherin in their domains of localization nor has any physical interaction between the two ever been documented. The manner in which Crumbs regulates adherens junctions still remains ambiguous.

Crumbs has been shown to regulate the cytoskeletal organization of the epithelium. Many lines of evidence suggest that *Crumbs* mutant epithelium have disorganized cytoskeleton (Letizia et al., 2011; Medina et al., 2002). This raises the chicken-egg question as to what is the primary defect in *Crumbs* mutants. Crumbs may directly regulate adherens junctions and cytoskeletal defects are secondary to the loss of epithelial integrity. Alternatively, Crumbs regulates the

cytoskeletal reorganization, which in turn facilitates the dynamics of adherens junctions. A third alternative is that it affects both processes independently.

Chapter 2 Poglut1 regulates mammalian Notch signaling during gastrulation

Introduction

To identify novel regulators of gastrulation EMT, we carried out an ENU (N-ethyl N-nitrosourea)-induced mutagenesis screen and identified mutants based on their gross phenotypic abnormalities. We isolated the *wing-shaped neural plate* (*wsnp*) mutant from one such screen as it had a disrupted morphology at mid-gestation (Garcia-Garcia et al., 2005; Kasarskis et al., 1998). The mutants were characterized by their shortened body axis and a flat neural epithelium, which failed to close and form a neural tube. The mutants were morphologically indistinguishable from the wild type until early head fold stage; the phenotype became apparent by late head fold stage and eventually dies around embryonic die 9.0.

2.1. Characterization of *wsnp* phenotype

2.1.1. Reduction of mesoderm-derived tissues.

The mutants are considerably shorter than wild type along the anterior-posterior axis at E8.5. Analysis with markers suggests that while all mesoderm derivatives are specified, they were reduced. The axial, paraxial, cardiac and nascent mesoderm were visualized by in situ hybridization.

Paraxial mesoderm can be visualized by *Meox1* expression, which is segmented into somites in wild type embryos at E8.5 (Figure 2.1A). *wsnp* mutants have reduced paraxial mesoderm, which fails to segment into somites. *Brachyury*, which is expressed in the axial mesoderm, shows a continuous midline in wild-type embryos at E8.5. *wsnp* mutants have a short and discontinuous midline, as

seen by breaks in *Brachyury* expression (arrow in Figure 2.1B). The nascent mesoderm emergent from the primitive streak can also be visualized by *Tbx6* expression, which extends along the proximal-distal axis of the wild type embryo at E8.5. Unlike wild type embryos, the domain of *Tbx6* expression was highly reduced in the mutants at E8.5 (Figure 2.1C), suggesting that the primitive streak failed to increase in length in the mutants. The failure to elongate the streak could also be visualized by *Brachyury* expression (Figure 2.1B), which shows a smaller primitive streak in the mutants at E8.5. *Nkx2.5*, a marker for cardiac mesoderm is also expressed in the mutants, but was morphologically different from wild type at E8.5 (Figure 2.1D). The mutants fail to fuse the lateral heart fields and form a single heart tube at later stages.

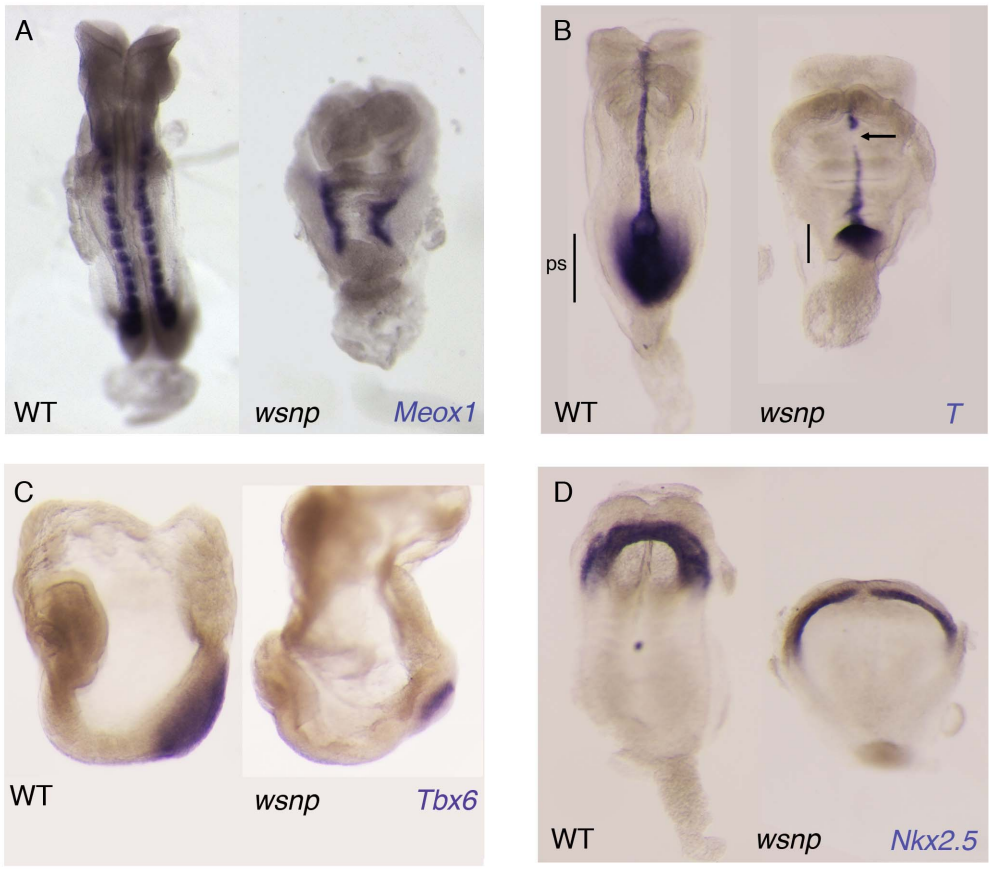


Figure 2.1 Defects in mesoderm derived tissues in *wsnp* mutants
In situ for expression of *Meox1, Brachyury, Tbx6 and Nkx2.5* (A,B,C and D). *Meox1* expression shows the reduction in paraxial mesoderm and its failure to segment into somites. *T or Brachyury* expression shows discontinuous notochord in *wsnp* mutants (B). *Tbx6*, expressed in nascent mesoderm is highly reduced in *wsnp* mutants (C). *Nkx2.5*, which is expressed in cardiac mesoderm, is morphologically different in *wsnp* mutants. A-dorsal view with anterior on top. B, D-ventral view with anterior on top. C-lateral view with anterior on left. Arrow in B shows break in Brachyury expression. Ps-primitive streak. Line indicates the length of the streak in B. Scale bars – 150 µm

In addition to the deficit in mesoderm-derived tissues, the embryos fail to close their neural tube. The defect in neural tube closure is apparent in transverse

sections through the neural tube immunostained for Sox2, a pan-neural marker (Figure 2.2). While wild type embryos have initiated the process of neural tube closure, the epithelium remains flat in *wsnp* mutants.

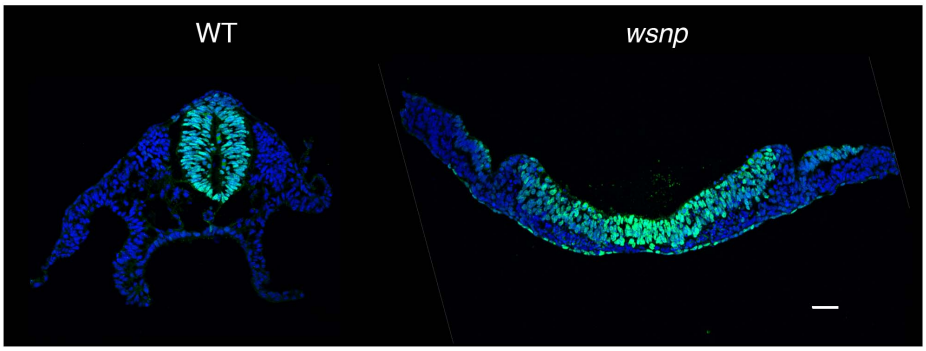


Figure 2.2 Defects in neuroepithelium in *wsnp* mutants
Immunostaining for Sox2 labeling the cells of the neural epithelium. While wild type beings to close the epithelium to form neural tube, the tissue remains flat in *wsnp* mutants. Scale bar: 42 μm.

2.1.2. Signaling networks at the primitive streak

The reduction in mesoderm-derived tissues led us to investigate the status of signaling pathways at the primitive streak. Mouse mutants that directly or indirectly affect the levels of Nodal have a gastrulation phenotype (Hart et al., 2002; Jin and Ding, 2013). To test whether Nodal levels were affected in *Poglut1*^{wsnp} we used the *Nodal-LacZ* transgene to visualize the levels of Nodal (Varlet et al., 1997). We generated wild type and mutants that carried a single copy of this transgene and visualized LacZ expression (by beta-galactosidase activity). In wild-type embryos, Nodal-LacZ is expressed in the primitive streak at E7.5 and extends along the proximal-distal axis of the embryo. *wsnp* mutants also

expressed Nodal at the primitive streak, although the domain of expression was smaller compared to wild type.

Mutants that establish a primitive streak but fail to maintain it like the *Wnt3a* mutants have defects in mesoderm-derived tissues. To test whether *wsnp* mutants had altered Wnt signaling, we generated wild type and mutants that carried a copy of TOPGAL reporter transgene. The TOPGAL transgene can be used to visualize the output of Wnt signaling because it has three LEF/TCF binding sites fused to the gene coding beta-galactosidase (DasGupta and Fuchs, 1999). Like Nodal, the LacZ is expressed in the primitive streak at E7.5 and extends till the distal tip of the embryo. *wsnp* mutants had a reduced domain of Wnt activity. These data suggest that although the mutants have normal Nodal and Wnt signaling which extends till the distal tip of the embryo, the primitive streak was smaller and failed to increase in length along the proximal-distal axis compared to wild-type at E8.5.

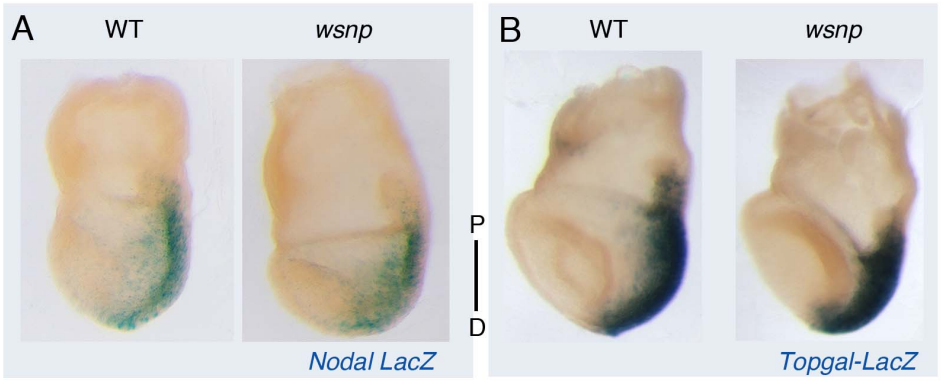


Figure 2.3 Nodal and Wnt signaling at the primitive streak.
Both Nodal-lacZ expression (A) and TOPGAL reporter activity (B) show that a single primitive streak is established at the posterior side of the embryo. However, it appears smaller in length along the proximal-distal axis when

compared with wild type at E7.5. Lateral views of the embryo with anterior to the left. Distal tip at the bottom.

2.2. *wsnp* is an allele of Poglut1

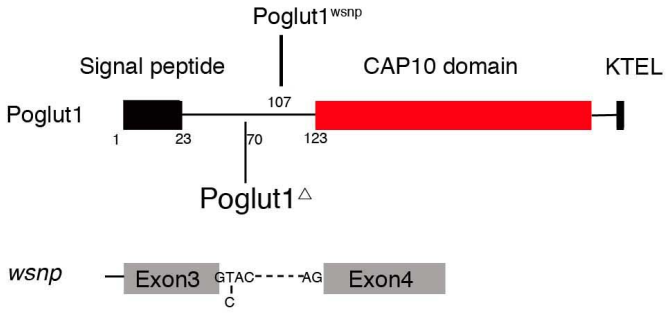


Figure 2.4 *wsnp* is an allele of Poglut1.
Protein domains of Poglut1 consist of a signal peptide, ER-retention signal and an enzymatic CAP10 domain. The splice mutation in *wsnp* allele leads to proteins with deletions in the enzymatic domain.

We mapped *wsnp* to a 407 kb interval between D16Mit90 and D16Mit12 SSLP markers and used next generation exome sequencing to identify sequence changes in the interval (see Methods for sequencing). A single nucleotide substitution was identified, a T to C transition in the splice donor site of intron 3 of Protein O-glucosyltransferase 1 (Poglut1), an enzyme that was shown to add O-glucose to Epidermal Growth Factor repeats of proteins in the endoplasmic reticulum (Shao et al., 2002). The protein consists of an enzymatic CAP10 domain and a four amino acid endoplasmic reticulum retention signal (KTEL) at its C-terminus. The *wsnp* mutation results in multiple abnormal spliced products (Figure 2.5) that lead to truncated or deleted proteins and would be predicted to act as a strong loss-of-function allele (Figure 2.4,2.5).

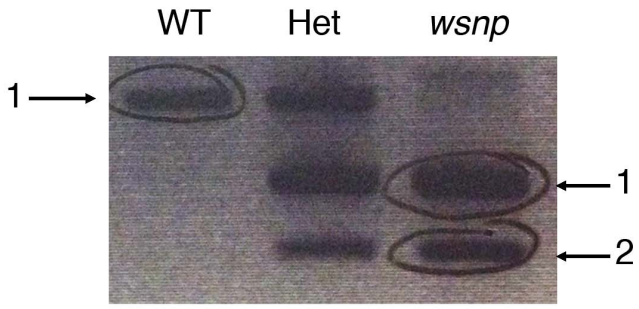


Figure 2.5 Abnormal splicing of *Poglut1*
RT-PCR analysis of RNA obtained from E8.5 wild type embryo, embryo heterozygous for *wsnp* and *wsnp* mutant embryo analyzed with primers to amplify exon1 to exon5 of *Poglut1*. Wild-type lane shows a single band, the mutants have around two different splice isoforms. The heterozygous embryos show all the bands. I sequenced the various bands. The following are the identity of the bands in the mutant: 1 and 2 – splice isoforms with deletion of exon3 leading to a protein with deletion in enzymatic domain.

To test whether *wsnp* is an allele of *Poglut1*, I generated embryos that were heterozygous for *wsnp* and a null allele of *Poglut1* (*Poglut1*$^{\Delta}$) (knock out first Eucomm allele- International Knock-out Mouse Consortium – Project 34189). This allele has exon 4 deleted, which causes a frame-shift that would cause a truncated protein. *wsnp*/*Poglut1*$^{\Delta}$ embryos were identical in phenotype to null/null and *wsnp/wsnp*, demonstrating that *wsnp* is a null allele of *Poglut1* (Figure 2.6). Like *wsnp* homozygotes, the trans-heterozygotes (*wsnp*/ *Poglut1*$^{\Delta}$) had reduced paraxial mesoderm, which failed to segment properly into somites, as assayed *Meox1* expression. The *Poglut1*^{gt} allele (knock out first Eucomm allele-International Knock-out Mouse Consortium – Project 34189) which has exon 4 flanked by LoxP sites and a LacZ coding region inserted in intron 3 leading to a truncated fusion protein, was also equivalent to a null allele, as *Poglut1*^{gt}

homozygous embryos resembled *wsnp* homozygotes and failed to complement *wsnp*.

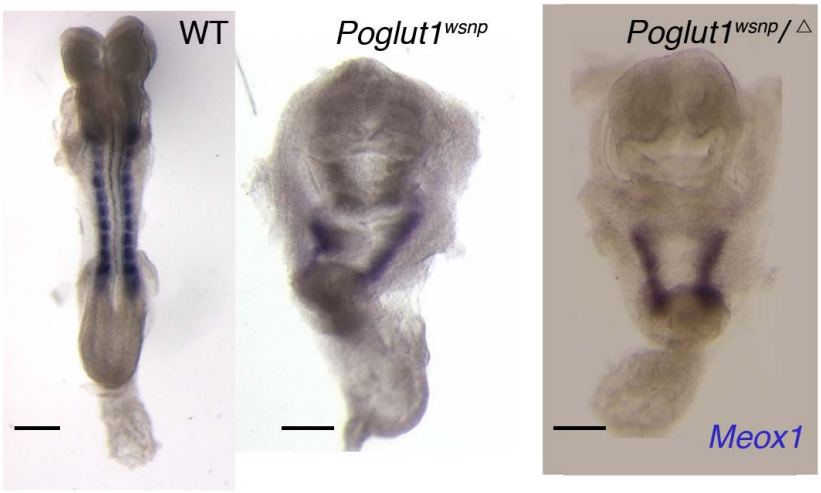


Figure 2.6 *wsnp* allele fails to complement *Poglut1*$^{\Delta}$
In situ for *Meox1* expression at E8.5 in *wsnp* mutants and trans-heterozygotes (*wsnp*/ *Poglut1*$^{\Delta}$) shows similar reduction in paraxial mesoderm and failure of segmentation of somites. Anterior is up. Scale bars- 150 μm

2.3. Poglut1 is ubiquitously expressed

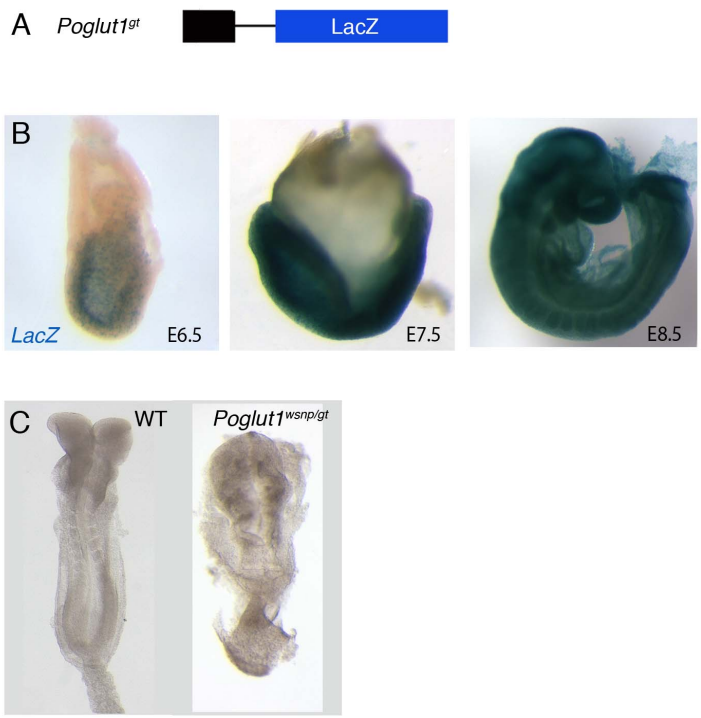


Figure 2.7 Expression of *Poglut1*
(A) The LacZ fusion protein coded by the *Poglut1*^{gt} allele
(B) Ubiquitous expression of *Poglut1* in the embryonic derived tissues from E6.5 to E8.5 seen by beta-galactosidase activity in *Poglut1*$^{gt/+}$ embryos.
(C) *Poglut1*^{gt} allele failed to complement *wsnp* allele. The trans-heterozygotes (*Poglut1*$^{wsnp/gt}$) have phenotype similar to *wsnp*.

Using the β-galactosidase activity of the *Poglut1*^{gt} allele, it appeared that the gene was expressed throughout the embryo from embryonic day 6.5 to 8.5 (Figure 2.7). Its expression was absent in extra-embryonic tissues.

2.4 Poglut1 activity is required in embryonic tissues

Figure 2.8 Conditional deletion of Poglut1 in embryonic tissues
Meox1 expression in wild type and *epiblast-deleted Poglut1* showing similar phenotype to *wsnp* and *Poglut1*$^{\Delta}$

To determine whether Poglut1 activity was required primarily in tissues of embryonic origin, I generated embryos with *Poglut1* deleted specifically in the epiblast using the *Sox2-Cre* transgene (Hayashi et al., 2002) and the conditional *Poglut1* allele. I found that *Poglut1 epiblast deleted* (*Sox2-Cre, Poglut1*$^{flox/\Delta}$) embryos died around E9. Phenotypically they resembled *wsnp* mutants at E8.5 with reduced paraxial mesoderm, unsegmented somites and flat neural epithelium (Figure 2.8). Thus Poglut1 activity is required in the epiblast-derived tissues for normal gastrulation.

2.5. Poglut1 activity is essential for mammalian Notch signaling

2.5.1. Down-regulation of active–Notch1 in *wsnp* mutants

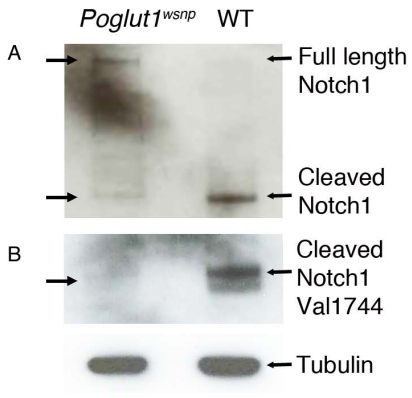


Figure 2.9 Reduction of cleaved-Notch1 levels in *wsnp* mutants.
Western blot analysis of protein extracts from wild type and *wsnp* mutant embryos at E8.5 probed for full length Notch1 (A) and cleaved-Notch1 (B). Antibody used in (A) recognizes both full length Notch1 and cleaved-Notch1. Upper arrows in (A) point to full length Notch1, which is increased in *wsnp* mutants, while lower arrows point to cleaved-Notch1, which is decreases in *wsnp* mutants compared to wild-type at E8.5. Antibody in (B) specifically recognizes the cleaved-Notch1 (arrows in B) and shows that the *wsnp* mutants have very low levels of cleaved-Notch1 compared to wild-type at E8.5. Tubulin was used as loading control.

Poglut1 was shown to affect Notch signaling in mammalian cell lines (Fernandez-Valdivia et al., 2011). However its role in mouse gastrulation was not investigated. To determine whether Notch signaling was altered in *Poglut1*^{wsnp} mutants, I performed western blots with lysates obtained from whole embryos at E8.5. I probed the blots with two antibodies, one that recognizes the intracellular domain of Notch1. Therefore it recognizes both full length and cleaved Notch1 (Figure 2.9A). The other antibody (Figure 2.9B) is specific to the residue exposed upon cleavage by gamma secretase (Val1744), therefore it recognizes the cleaved or active Notch1. I found that Notch pathway was active at this embryonic stage

as seen by the expression of cleaved Notch1 in wild-type embryos at E8.5 (Figure 2.9A,B). Both the antibodies showed that cleaved Notch1 was dramatically reduced in *Poglut1*^{wsnp} mutants. Conversely, I found an increase in full length Notch1 in *Poglut1*^{wsnp} mutants compared to wild type (Figure 2.9).

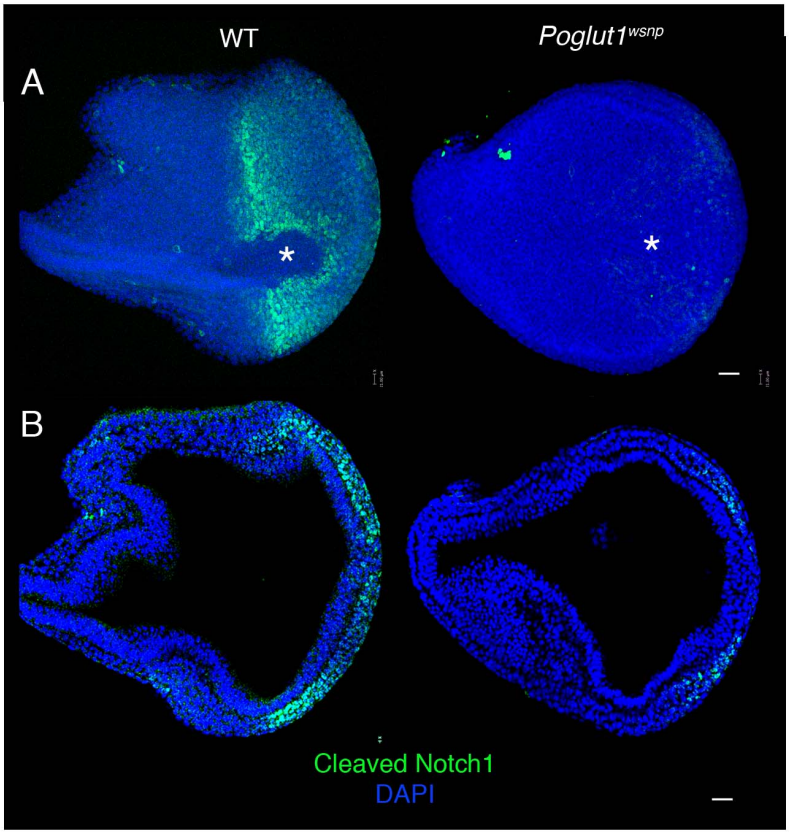


Figure 2.10 Down-regulation of cleaved Notch1 expression in of *Poglut1*^{wsnp} mutants
Whole mount immunostaining for cleaved Notch1 at E7.75. Extended projection of view from the distal tip of the embryo shows cleaved Notch1 expression around the node in wild type embryos, which was absent in *Poglut1*^{wsnp} mutants (A). Single optical section of transverse section through the primitive streak showing cleaved Notch1 expression in the nascent mesoderm in wild type embryo and its reduction in *Poglut1*^{wsnp} mutants (B). Distal views of the embryo. with anterior on the left in (A). Anterior is on the left in transverse sections in (B). * denotes the position of the node. Scale bar - 42 μm

Notch1 and its ligands are expressed during gastrulation (Bettenhausen et al., 1995; Dunwoodie et al., 1997; Przemeck et al., 2003) and cleaved Notch1 is expressed in the nascent mesoderm from E7.0 (Del Monte et al., 2007). Using whole mount antibody staining with cleaved Notch1 antibody, we probed for the expression pattern in *Poglut1*^{wsnp} mutants. Notch1 was active around the node (Figure 2.10A) and in the nascent mesoderm cells emerging from the primitive streak in wild type embryos (Figure 2.10B). The activity was highest in cells emerging from the streak, followed by a slight down-regulation and then increase again as we move away from the streak. This pattern probably reflects the role of Notch signaling in the somite segmentation clock and is therefore preparing the nascent mesoderm to segment into future somites. Active Notch1 was not detected in *Poglut1*^{wsnp} mutants in both the distal view and the transverse section through the primitive streak (Figure 2.10B).

2.5.2. Down-regulation of Notch target genes

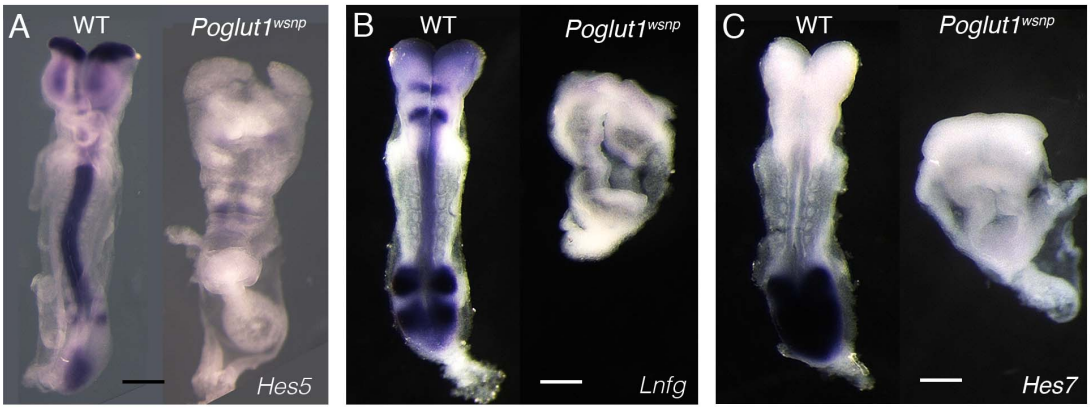


Figure 2.11 Down-regulation of Notch pathway in *Poglut1*^{wsnp} mutants
In situ hybridization for the expression of *Hes5*, *Lunatic Fringe* and *Hes7*, downstream targets of Notch activation, at E8.5. All targets are expressed in the pre-somitic mesoderm in wild type and have been implicated in the somitogenesis. *Poglut1*^{wsnp} mutants completely lack expression of any of these target genes. Ventral view (A) and dorsal view (B, C) of the embryos with anterior on top. Scale bar – 150 µm

In mammals, the Hes and Hey family of transcription factors are direct targets of Notch signaling. To determine whether Notch signaling is down-regulated in *Poglut1*^{wsnp} mutants I looked at the transcription targets of Notch pathway. Consistent with the westerns and immunostaining, I found that direct transcriptional targets of Notch pathway, *Hes5*, *Lunatic Fringe* and *Hes7* (Figure 2.11A,B and C respectively) were strongly reduced in *Poglut1*^{wsnp} mutants compared to wild type. These genes are primarily involved in segmentation of the somites. These observations indicate that *Poglut1*^{wsnp} mutants have negligible levels of Notch signaling and this loss of Notch signaling in *Poglut1*^{wsnp} mutants probably leads to failure of segmentation of the paraxial mesoderm.

In addition to loss of *Hes* gene expression, the expression of *Delta-like1*, which is normally inhibited by Notch, was ectopically expressed in *Poglut1*^{wsnp} mutants (Figure 2.12). Similar ectopic expression of *Delta like1* has been observed in *RbpjK* mutants and *Presenilin* double mutants (de la Pompa et al., 1997; Donoviel et al., 1999).

Figure 2.12 Ectopic expression of *Dll1* in *Poglut1*^{wsnp} mutants
While the *Hes* genes are down regulated, *Dll1* is ectopically expressed in the neural epithelium at E8.5. Dorsal view of embryos with anterior on top.

2.5.3. Role of Notch signaling in gastrulation EMT

To determine whether *Poglut1*^{wsnp} mutant phenotype is a result of reduction in Notch signaling, I tried to rescue the mutants by overexpressing active-Notch1 in embryonic tissues of the mutants. I generated embryos over-expressing active-Notch1 specifically in the epiblast using the *Sox2-Cre* transgene and *R26-NICD* transgene (Hayashi et al., 2002; Murtaugh et al., 2003). Embryos that over-expressed active Notch1 had an expanded domain of Brachyury expression

(Figure 2.13). The overexpression of active-Notch1 by itself had a severe gastrulation phenotype at E7.5 thereby obscuring interpretation in the mutants.

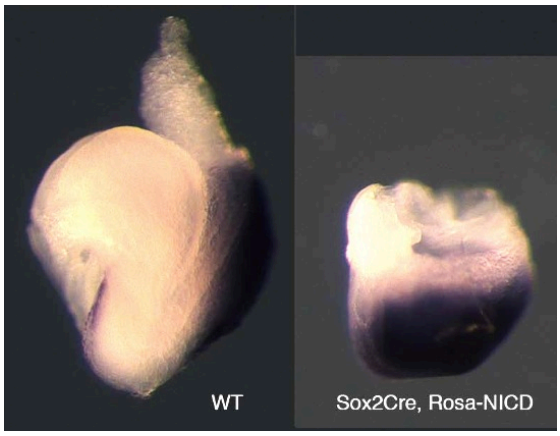


Figure 2.13 Over-expression of active-Notch1 affects gastrulation
Brachyury in situ on wild type and embryos over-expressing NICD in embryonic tissues at E8.5 shows massive expansion of *Brachyury* expression in mutants.

2.6. Notch independent roles of Poglut1

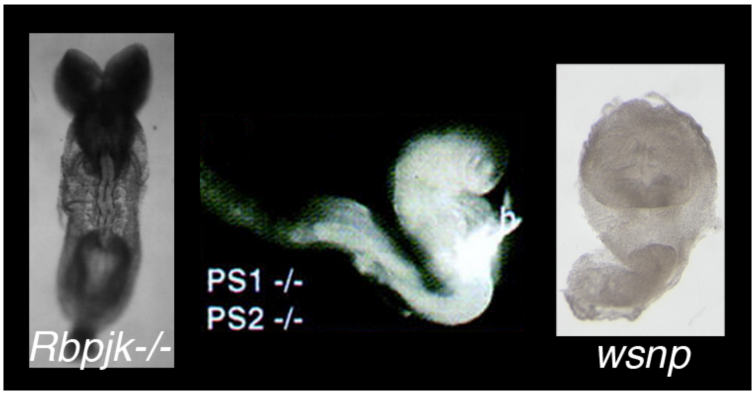


Figure 2.14 Comparison of *Poglut1*^{wsnp} phenotype with canonical Notch pathway mutants
RbpjK $^{-/-}$ mutants and embryos lacking *Presenillins 1* and *2* have additional phenotypes not described for *Notch1* mutants such as delay in closure of anterior neuropore and reduction in midbrain mesenchyme. However they make considerable amount of mesoderm derived structures compared to *Poglut1*^{wsnp}. Adapted from (Donoviel et al., 1999; Oka et al., 1995)

Despite the apparent role of Poglut1 in regulating Notch signaling, the phenotypes of these mutants do not resemble any Notch pathway mutants. While mammals have four Notch proteins and multiple ligands, no one has analyzed a quadruple Notch mutant. However, mammals have a single transcription factor *Rbpjk* and two *Presenillins*. Although mouse mutants for *Rbpjk* and *Presenillin* double mutants are stronger than single *Notch1* mutants, they are still more normal than *Poglut1*^{wsnp} mutants (Figure 2.14) (Donoviel et al., 1999; Oka et al., 1995). *Presenilin* double mutants die around e9.5 due to cardiovascular defects and make considerable amounts of mesoderm and mesoderm-derived structures compared to *Poglut1*^{wsnp} (Donoviel et al., 1999). Although *Rbpjk* mutants die around E8.5, they do make some poorly condensed and disorganized somites

unlike *Poglut1*^{wsnp} (Oka et al., 1995). Thus Poglut1 must have a Notch-independent activity in the early mouse embryo.

2.7. Discussion:

Drosophila Rumi was identified as a regulator of Notch signaling. Loss of *Drosophila Rumi* phenocopies loss of Notch mutants in a temperature sensitive manner. Rumi was shown to function as an O-glucosyltransferase rather than a chaperone for Notch (Acar et al., 2008). Studies in Drosophila and mammalian cell lines indicate that O-glucosylation does not affect the ligand binding to Notch1 and that Rumi affects Notch signaling at a step between ligand binding and S3 cleavage (Fernandez-Valdivia et al., 2011). Similarly, we see that loss of O-glucose leads to almost complete reduction of S3 cleaved Notch1 and therefore Notch signaling. Notch signaling is involved in segmentation of the pre-somitic mesoderm (Wahi et al., 2014). The expression pattern of active Notch1 at the primitive streak indicates its potential role in setting up the segmentation clock. The complete absence of Notch signaling in *Poglut1*^{wsnp} mutants suggests that the segmentation clock is not activated in these mutants. This is consistent with the phenotype of *Poglut1*^{wsnp} mutants, as they fail to segment the paraxial mesoderm into somites.

The Notch ECD is extensively decorated with both O-fucose and O-glucose, which are further extended with other sugars. My data suggests that O-glucosylation of mammalian Notch1 is essential for the activation of Notch signaling during development. In mammals while loss of *Pofut1* phenocopies *Rbpjk* mutants (Okamura and Saga, 2008), *Poglut1* mutants have a more severe phenotype

suggesting that besides regulating Notch signaling Poglut1 has additional biologically relevant targets during development.

Chapter 3 Poglut1-dependent O-glucosylation of extracellular domain of Crumbs2 is required for its function

Introduction

Despite the defects observed in Notch signaling in *Poglut1*^{wsnp} mutants, the phenotype of *Poglut1* mutants was more severe than canonical Notch pathway mutants (Figure 2.14). This suggested that Poglut1 had other biologically relevant targets during development. Poglut1 adds O-glucose to C1-X-S-X-A/P-C2 consensus sequence in EGF repeats of proteins in the endoplasmic reticulum (Rana et al., 2011; Shao et al., 2002). To identify other potential targets of Poglut1, I used the Prosite database to search for proteins containing this consensus sequence (Table 3.1). Among the proteins in the list, Crumbs family of proteins was a likely candidate owing to the resemblance of the *Poglut1*^{wsnp} phenotype to *Crumbs2* mutant phenotype. At the beginning of this thesis project, the *Crumbs2* mutant phenotype had not been published. Instead, I considered Crumbs proteins as strong candidates because of the high resemblance of *Poglut1*^{wsnp} phenotype to another ENU-induced mutant called *lulu* harboring a point mutation in a FERM domain containing protein Epb4.1l5 that was shown to interact with the intracellular domain of Crumbs (Gosens et al., 2007; Laprise et al., 2009; Lee et al., 2007).

Table 3.1 List of mouse proteins containing Poglut1 consensus sequence

Notch (1,2,3,4)	Celsr (1,2,3)	Agrin
Delta (1,3 and 4)	Fat 4	Crumbs (1,2)
Jagged (1 and 2)	Amaco	Cubulin
Delta Notch like EGF repeat containing protein	Neurexin 1	Fibrillin (1,2)
Delta like (1 and 2)	Fibulin 7	SNED1
Factor (VII, IX, X)	Slit (1,2,3)	Thrombospondin (1,2,4)
LDL receptor 2	Hepatocyte growth factor activator	PAMR1
CD93	Multimerin	Protein Z
Hyaluronan binding protein 2	Polydom	Versican

3.1 Domain organization of Mammalian Crumbs

The mammalian Crumbs family of proteins consists of three members, Crumbs1, Crumbs2 and Crumbs3. Only Crumbs1 and Crumbs2 have EGF repeats on their extracellular domain that can be modified by Poglut1 (Figure 3.1).

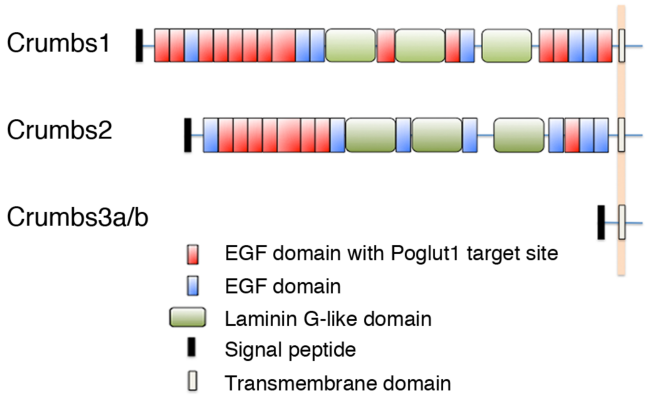


Figure 3.1 The domain organization of mammalian Crumbs proteins. Crb proteins have a short highly conserved cytoplasmic domain and large variable extracellular domain. Crumbs1 has 19 EGF repeats of which 13 can be

potentially modified by Poglut1, while Crumbs2 has 15 EGF repeats of which 8 can be modified by Poglut1.

3.2 O-glycosylation of Crumbs2 in mouse embryos

Proteins that are modified by sugars have altered migration on SDS polyacrylamide gels (PAGE). In *Poglut1*^{wsnp} mutants, the loss of these sugar modifications might alter the migration of Crumbs2. In order to determine whether Crumbs2 was a target for Poglut1, I looked at the migration of Crumbs2 on SDS-PAGE. I found that the migration of Crumbs2 was altered in *Poglut1*^{wsnp} mutants, consistent with the hypothesis that loss of Poglut1 leads to loss of sugar modifications on Crumbs2 (Figure 3.2).

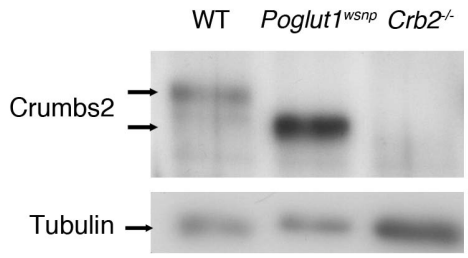


Figure 3.2 Altered migration of Crumbs2.
Western blot of wild type, *Poglut1*^{wsnp} and *Crumbs2*$^{-/-}$ mutant embryo lysates at E8.5 probed with a pan-Crumbs antibody, showing the more rapid migration of Crumbs2 in *Poglut1*^{wsnp} embryos.

To determine whether the sugar modification was essential for the expression and/or localization of the protein, I looked at the expression of Crumbs2 in embryo sections. Crumbs2 was expressed in the neural epithelium and localized to the apical region of the epithelium in wild type embryos at E8.5 (Figure 3.3A). However, in *Poglut1*^{wsnp} mutants this apical enrichment was lost (Figure 3.3B).

Crumbs2 was also localized to the apical membrane in the primitive streak of wild type embryos (Figure 3.3C). Similar to the neural epithelium, the apical membrane localization of Crumbs2 was lost in *Poglut1*^{wsnp} mutants (Figure 3.3D).

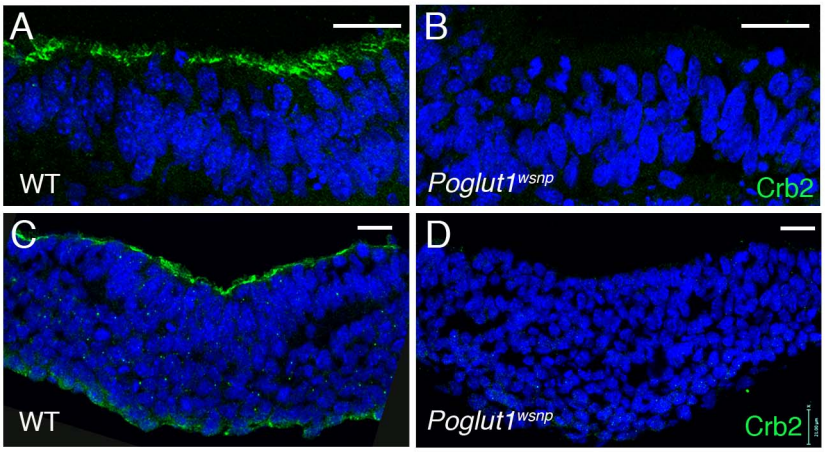


Figure 3.3 O-glycosylation is essential for membrane localization of Crumbs2 Transverse section of wild type (A) and *Poglut1*^{wsnp} (B) neural epithelia showing Crumbs2 localization by immunofluorescence at E8.5. Apical membrane enrichment of Crumbs2 in the neural epithelium is lost in *Poglut1*^{wsnp} mutant embryos. Transverse sections of wild type (C) and *Poglut1*^{wsnp} (D) primitive streak showing Crumbs2 localization at E8.5. Similar to the neural epithelium, apical membrane localization of Crumbs2 is lost in the primitive streak of *Poglut1*^{wsnp} mutant embryos. Scale bar – 21 μm.

In contrast to the surface localization of Crumbs2, Crumbs1 was detected only in the Golgi at this stage, and this expression is unaltered in *Poglut1*^{wsnp} mutants (Figure 3.4A). To test whether the *Poglut1*^{wsnp} phenotype was a result of loss of both Crumbs1 and Crumbs2 function, I generated *Crumbs1, 2* double mutants and found that these double mutants resemble *Crumbs2* mutants, suggesting that Crumbs1 was dispensable during mammalian gastrulation (Figure 3.4B).

Together these data indicate that sugar modifications are absent on Crumbs2 in *Poglut1*^{wsnp} mutants and that O-glucosylation is essential for membrane localization of Crumbs2.

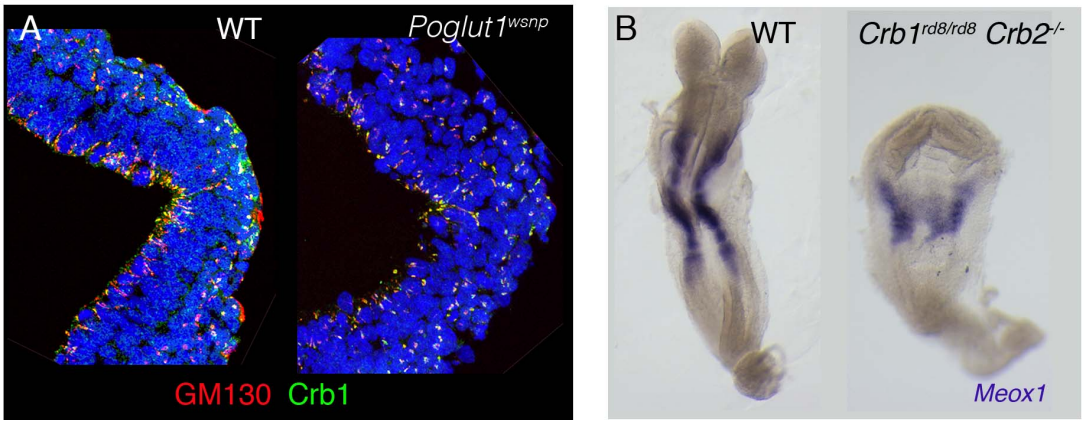


Figure 3.4 Crumbs1 does not regulate gastrulation
(A) Expression of Crumbs1. Immuno-staining for Crumbs1 (green) and GM130 (a golgi marker in red) in transverse sections through the primitive streak of wild type and *Poglut1*^{wsnp} embryos at E8.5. Crumbs1 is expressed in the mouse primitive streak and is localized to the Golgi. Its expression is not altered in *Poglut1*^{wsnp} mutants at this stage. (B) In situ hybridization for *Meox1* expression in *Crumbs1*$^{rd8/rd8}$, *Crumbs2*$^{-/-}$ double mutants. The double mutants have a phenotype similar to *Crumbs2*$^{-/-}$ mutants such as reduction in paraxial mesoderm and failure to segment mesoderm properly into somites, thus suggesting that Crumbs1 does not play a crucial role in mammalian gastrulation. Dorsal view of embryos with anterior on top.

3.3. Modification of Crumbs2 by Poglut1 in embryoid bodies

3.3.1. Altered migration of Crumb2 in embryoid bodies

To determine whether Crumbs2 was a biologically relevant direct target of Poglut1, I wanted to test biochemically whether Crumbs2 was glucosylated *in vivo*. ES cells do not express endogenous Crumbs2 and were therefore not appropriate for analysis. Mouse ES cells can be differentiated to embryoid bodies, which recapitulate some of the stages of mouse gastrulation (Leahy et al., 1999) and can be isolated in large enough quantities for biochemical analysis (Kurosawa, 2007). When cultured in the absence of 2i and LIF in low-adherent bacterial dishes, mouse ES cells differentiate to form embryoid bodies. By western blot analysis I found that embryoid bodies start expressing endogenous Crumbs2 between day 5 day 6 of differentiation, which is comparable to early gastrulation of mouse embryos (Figure 3.5). I therefore derived wild type and *Poglut1*^{wsnp} mutant embryonic stem cells from blastocysts using the 2i method (Silva et al., 2008). As in the embryos, I observed more rapid migration of Crumbs2 in *Poglut1*^{wsnp} embryoid bodies compared to wild type (Figure 3.5) and therefore used embryoid bodies for biochemical analysis.

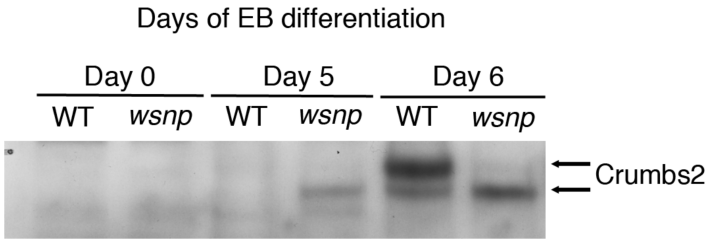


Figure 3.5 Glycosylation of Crumbs2 in Embryoid bodies
Expression of Crumbs2 in embryoid bodies. Western blot for expression of Crumbs2 shows that EBs begin expressing Crumbs2 protein between day 5 and

day 6 of differentiation from embryonic stem cells. I observed the characteristic shift in migration of Crumbs2 in EBs generated from *Poglut1*^{wsnp} at day 6.

3.3.2. O-glucosylation of EGF repeat 6 of tagged full length Crumbs2

I generated stable ES cells lines expressing tagged full length Crumbs2 from both wild type and *Poglut1*^{wsnp} mutants. Crumbs2 was tagged with V5-and His at its C-terminal, as V5 epitope was not endogenously expressed in EBs and could be utilized to purify the protein efficiently. To determine whether Poglut1 modifies the extracellular domain of Crumbs2, I purified full-length Crumbs2 from wild-type (Poglut1+) and *Poglut1*^{wsnp} (Poglut1-) embryoid bodies differentiated for 2 days (day 2). To perform mass spectrometry analysis, we required large quantities (2 μg) of purified proteins from both wild type and mutants EBs. ES cells were cultured in a large scale (approximately 6 X 15cm plates). They were then induced to differentiate into EBs for 2 days and harvested for protein purification. The tagged Crumbs2 protein from *Poglut1*^{wsnp} embryoid bodies migrates faster compared to the wild type (Figure 3.6A). We cut out both bands (stained with gel code blue stain reagent), subjected them to chymotrypsin digest.

In collaboration with Beth Harvey from the lab of Robert Haltiwanger (SUNY, Stony Brook), we probed the resulting peptides for glycosylation using ionizing mass spectrometry. EGF-repeat 6 of Crumbs2 purified from wild type embryoid bodies shows a characteristic O-glucose-xylose-xylose trisaccharide at serine 271 (Figure 3.6B). Following the addition of O-glucose, it is extended into this trisaccharide in all proteins including Notch EGF repeats in which it is present (Acar et al., 2008; Fernandez-Valdivia et al., 2011). This sugar modification was

absent in peptides from Crumbs2 purified from *Poglut1*^{wsnp} embryoid bodies. Instead, the unmodified peptide was the major species obtained in the *Poglut1*^{wsnp} mutants (Figure 3.6C). Surprisingly, both the upper (slow migrating) and lower bands (fast migrating) in the wild type had the sugar modification, while no sample from the mutant protein was ever modified. We speculate that the altered migration could be cumulative effect of sugar modifications by Poglut1 on multiple EGF repeats. This data demonstrate that EGF repeat 6 on the extracellular domain of Crumbs2 is glucosylated by Poglut1 in vivo during mammalian development.

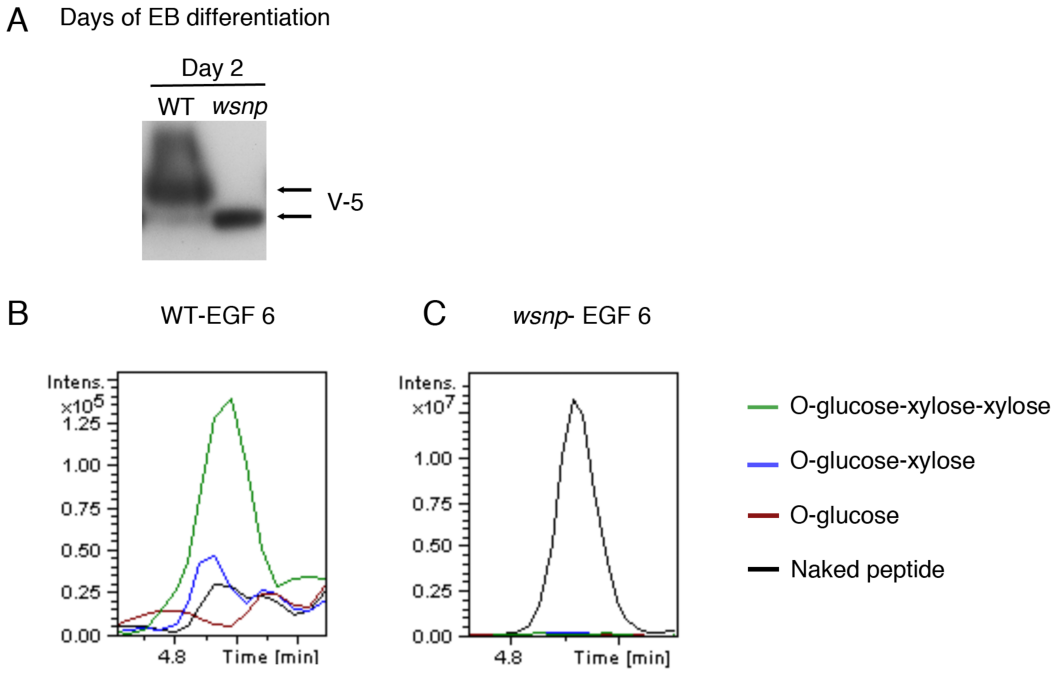


Figure 3.6 Glycosylation of EGF repeat 6 of tagged Crumbs2
Western blot for the migration of purified tagged Crumbs2 extracted from WT and *Poglut1*^{wsnp} ES cells expressing tagged full length Crumbs2 differentiated to EBs probed with V5 antibody. The differentiation enriched the glycosylation of Crumbs2 as observed by the shift in migration of V5-tagged Crumbs2 (A). Extraction ion chromatogram of peptides from EGF repeat 6 peptide of tagged Crumbs2 from WT (B) and *Poglut1*^{wsnp} (C) EBs differentiated for 2 days. The WT chromatogram shows the presence of trisaccharide O-glucose-xylose-xylose. The naked peptide without the initial O-glucose is the only species detected in the EIC from *Poglut1*^{wsnp}. I prepared the samples, ran the gels and cut the bands for mass spectrometric analysis. Beth Harvey from the lab of Robert Haltiwanger (SUNY Stony Brook) performed the mass spectrometry and analyzed the spectra.

3.4. *Poglut1*^{wsnp} and *Crumbs2* have nearly identical embryonic phenotypes

3.4.1. Morphological similarities

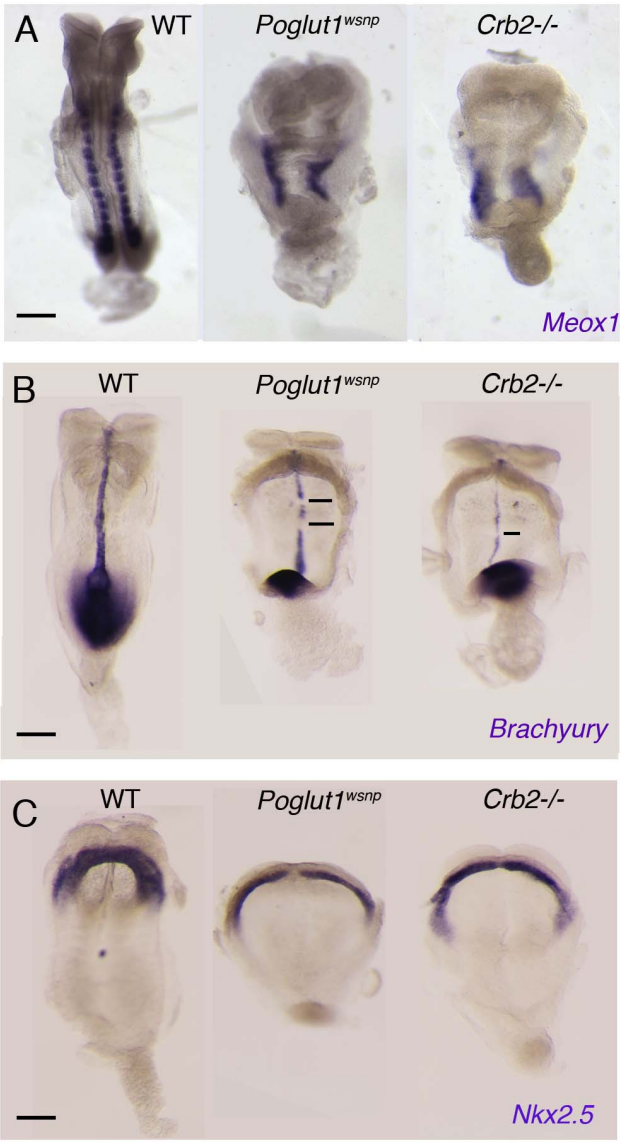


Figure 3.7 Similar phenotypes of *Poglut1*^{wsnp} and *Crumbs2* mutants
In situ for *Meox1* expression (A) shows the reduction in paraxial mesoderm in both mutants at E8.5 (dorsal view). In situ for *Brachyury* expression (B) at E8.5 shows a smaller primitive streak at the posterior end and the discontinuous midline in the mutants compared to wild type (ventral view, anterior up). In situ for *Nkx2.5* expression shows abnormal shape of the heart fields in the mutants at E8.5. Scale bar- 150 µm.

To determine whether the *Poglut1*^{wsnp} phenotype was caused by loss of Crumbs2 function, I compared the phenotypes of null alleles of the two genes. *Crumbs2* mutants arrest at mid gestation and look morphologically similar to *Poglut1*^{wsnp} mutants. Analysis with markers for gene expression revealed that while mesoderm structures were specified, there was a reduction in the amount of mesoderm in the mutants. This was prominent in the paraxial mesoderm. *Meox1*, which marks the paraxial mesoderm in the wild types, was highly reduced in the mutants (Figure 3.7A). *Brachyury*, an axial mesoderm marker was expressed in the midline in wild type embryos at E8.5. Both *Poglut1*^{wsnp} and *Crumbs2* mutants had a discontinuous midline as seen by *Brachyury* expression (Figure 3.7B). While cardiac mesoderm was specified in *Crumbs2* and *Poglut1* mutants as seen by *Nkx2.5* expression, its expression was morphologically different from wild type at E8.5 (Figure 3.7C).

3.4.2 Identical gastrulation defect in *Poglut1*^{wsnp} and *Crumbs2-/-* mutants

The most prominent phenotype of *Poglut1*^{wsnp} is the shortened body axis accompanied by a deficit of mesoderm. Mesoderm cells arise during gastrulation from an organized Epithelial-to-Mesenchymal Transition (EMT) at the posterior signaling center called the primitive streak. During EMT, epithelial cells lose their polarization and adopt motile mesenchymal characteristics. The gastrulation EMT requires breakdown of basement membrane and down-regulation of apical adherens junctions. Since *Crumbs2* mutants have defects at the primitive streak (Xiao et al., 2011), I wanted to determine whether the deficit of mesoderm in *Poglut1*^{wsnp} is a result of a defect in EMT at the primitive streak. Laminin, a component of the extracellular matrix must be broken down at the streak for cells to exit following EMT (Figure 3.8A, D, D'). E-cadherin, an adherens junction

marker is expressed apically in the epiblast and down regulated following EMT in the invaginating cells in wild type (Figure 3.8A, D, D''). I found that both *Poglut1*^{wsnp} and *Crumbs2* mutants are comparable to wild-type at E7.5. However by E8.0 both the mutants have a broader streak, marked by laminin breakdown. In addition, cells near the streak have some ectopic apical laminin expression (Figure 3.8E'). I found that there is an accumulation of cells expressing E-cadherin at the streak in *Poglut1*^{wsnp} mutants (Figure 2.8E''). E-cadherin expression is eventually down regulated, as some cells do migrate away from the streak. The *Crumbs2-/-* embryos also have a broader streak and an accumulation of cells expressing E-Cadherin at the primitive streak (Figure 3.8F, F', F''). The dearth of mesoderm cells is apparent in transverse sections with laminin staining. In wild-type embryos, we can observe the anterior head mesenchyme below the neural epithelium (Figure 3.8D). In both the mutants, there is very little anterior head mesenchyme (Figure 3.8E, F).

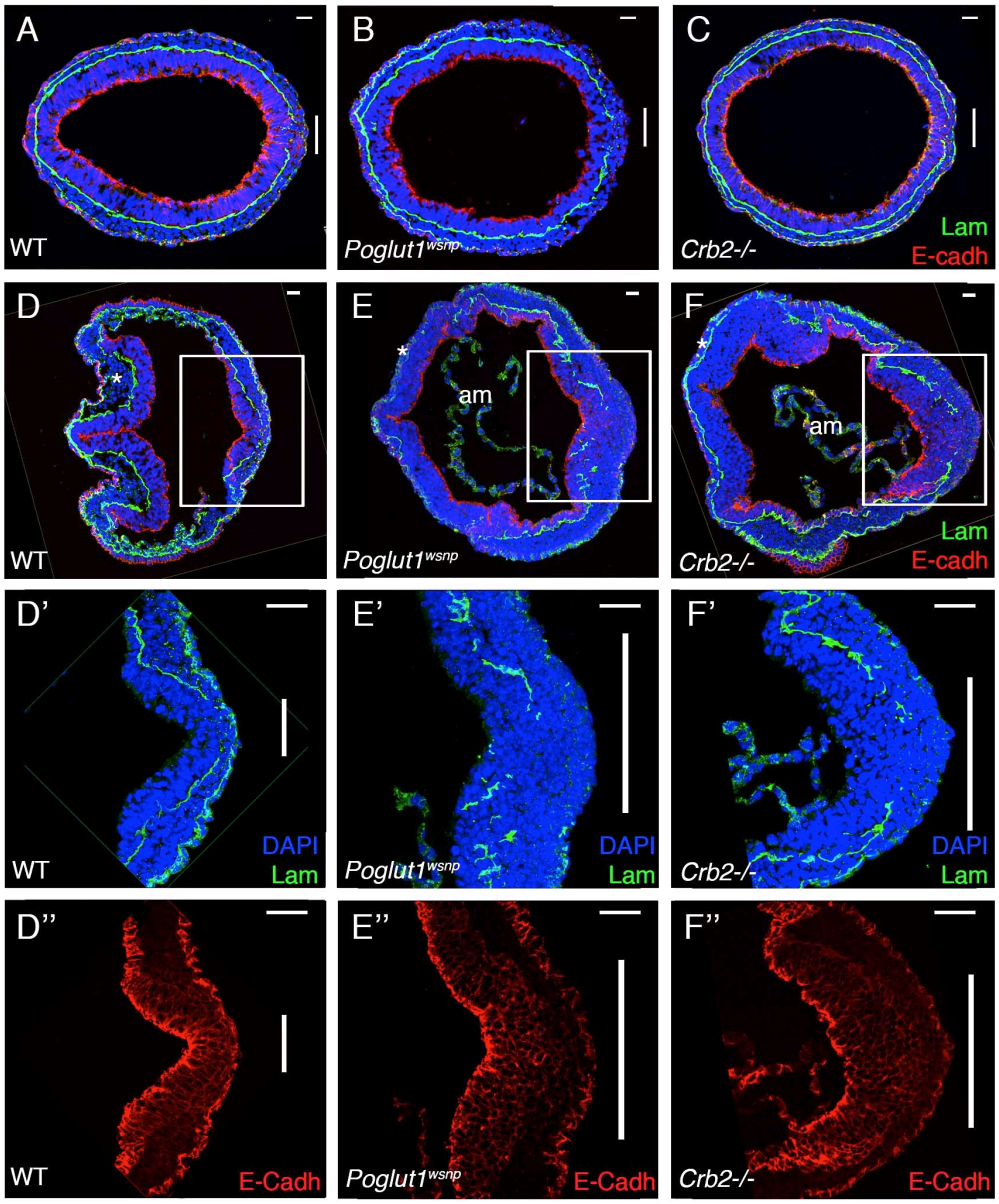


Figure 3.8 Gastrulation defects in *Poglut1*^{wsnp} and *Crumbs2*$^{-/-}$ embryos
Transverse sections through the wild type (A,D), *Poglut1*^{wsnp} (B,E) and *Crumbs2*$^{-/-}$ (C,F) embryos at E7.5 early bud (A,B,C) and E8.0 (D,E,F) immuno-stained for Laminin (green) and E-Cadherin (red). Both the mutants look comparable to wild type at the early bud stage. The gastrulation defect becomes more prominent at E8.0 where the mutants have lesser mesoderm and disorganized epithelium (compare head mesenchyme in WT compared to a single file of cells in both mutants, asterisk). D',E' and F' and D'', E'' and F'' are higher magnification of the primitive streak showing laminin expression (D',E' and F') and E-Cadherin (D'',E''and F''). Both mutants have broader streak as seen by laminin breakdown. Both *Poglut1*^{wsnp} and *Crumbs2*$^{-/-}$ mutants have an accumulation of cell expressing

E-cadherin, an adherens junction marker at the primitive streak. am-amnion, asterisk - head mesenchyme; line marks the width of the primitive streak. Scale bars – A to F - 21 μm and D' to F''- 41 μm

3.4.3 Identical neural differentiation phenotypes of *Crumbs2* and *Poglut1*^{wsnp} ES cells

ES cells can be differentiation into the neural lineage when cultured as a monolayer with N2B27 supplement at day 10 as seen by Tuj1 expression (Figure 3.9). ES cells with stable *Crumbs2* knock down were unable to differentiate into neural lineage as the rosette-like structures formed during neural differentiation failed to stabilize apical polarity proteins and therefore died (Boroviak and Rashbass, 2011). Similarly, *Poglut1*^{wsnp} ES cells died at the onset of neural specification and failed to differentiate into neurons seen by the absence of expression of Tuj1 (Figure 3.9). These data suggests that *Poglut1* ES cells behave like *Crumbs2* knock down ES cells and that sugar modification of Crumbs2 is essential for its function not just in gastrulation but also in other cell types.

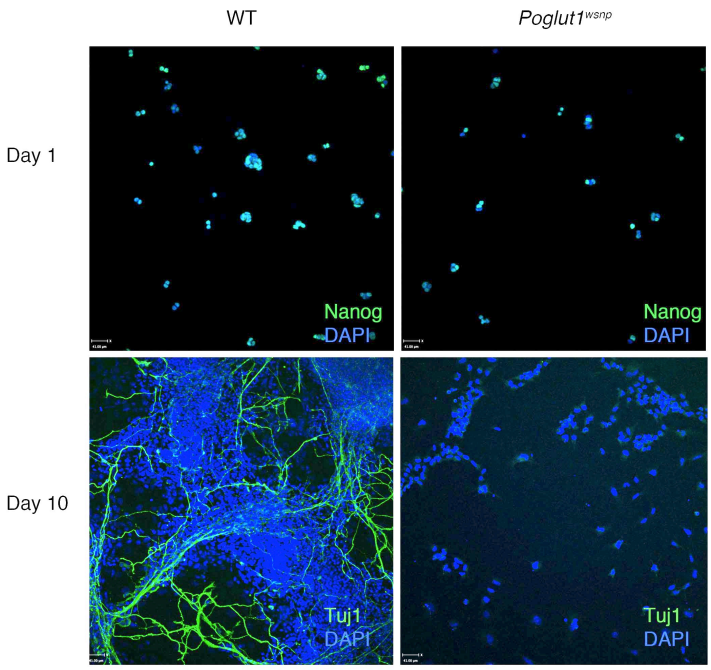


Figure 3.9 *Poglut1*^{wsnp} ES cells fail to undergo neural differentiation.
Wild type and *Poglut1*^{wsnp} ES cells initially expressing Nanog (day1) were plated at equal density and cultured as monolayers in N2B27 media. While WT ES cells start differentiating into neural lineage, *Poglut1*^{wsnp} ES cells start dying around day4/5 and fail to differentiate. Notice very few cells left behind at day 10 in *Poglut1*^{wsnp} cultures compared to wild type (compare DAPI positive nuclei in wild type and mutant at day 10)

3.5. Crumbs is not a biologically relevant target of Poglut1 in the *Drosophila* embryo

At the beginning of this thesis project, we were trying to determine whether Poglut1 modifies Crumbs proteins. The phenotype of mouse *Crumbs2* was not published then. While we were acquiring mouse lines for generating *Crumbs2* mutants, I decided to investigate the potential glycosylation of *Drosophila* Crumbs.

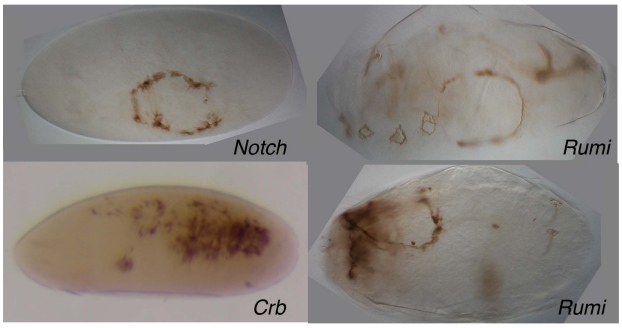


Figure 3.10 Cuticle preparations of *Notch*, *Crumbs* and *Rumi* mutant embryos grown at 29°C.
Rumi mutant embryos had ventral holes in their cuticle and therefore resembled *Notch* mutants rather than *Crumbs* mutants.

Rumi mutants are null alleles of Drosophila *Poglut1* and have a temperature sensitive effect on Notch signaling (Acar et al., 2008). At higher temperatures, they have a loss of Notch signaling phenotype. The cuticle phenotype of loss of Notch signaling is a ventral hole, due to the conversion of ventral ectoderm to a neural fate. *Drosophila* embryos lacking Crumbs, fail to form a proper epithelium, which eventually falls apart. As a result only crumbs of cuticle are left behind. To determine whether glycosylation of Crumbs is relevant in flies, we looked at the cuticle preparations of *Rumi* embryos at higher temperatures. I found that the cuticle preparations predominantly reflect loss of Notch signaling (Figure 3.10). A recent report suggests that *Drosophila* Crumbs can be glycosylated (Haltom et al., 2014). However, flies with a knock-in allele of Crumbs mutated in all O-glucosylation sites, were viable and did not have any major phenotypes. This suggests that, similar to our observations with the embryonic cuticle

preparations, O-glucosylation of Crumbs is not essential for its function in *Drosophila*.

3.6. A difference between the *Poglut1*^{wsnp} and *Crumbs2* phenotype

By gross morphology, *Poglut1*^{wsnp} and *Crumbs2* mutants have similar phenotypes. The main difference between them is that *Poglut1*^{wsnp} mutants have very low levels of Notch signaling, while the levels are unaffected in *Crumbs2* mutants (Figure 3.11A). As a read out of Notch activation, the paraxial mesoderm fails to initiate the somitogenesis clock and therefore remains unsegmented in *Poglut1*^{wsnp} mutants. However, since Notch signaling is active in *Crumbs2* mutants, they might have some level of segmentation of somites. *Uncx4.1* is a homeobox containing transcription factor, which is expressed in the posterior domain of somites in wild type embryos at E8.5. Its expression was completely absent in *Poglut1*^{wsnp} embryos, while its expression was maintained in *Crumbs2* mutants (Figure 3.11B). Although *Crumbs2* mutants express *Uncx4.1*, the somites were not completely normal and well condensed like wild type.

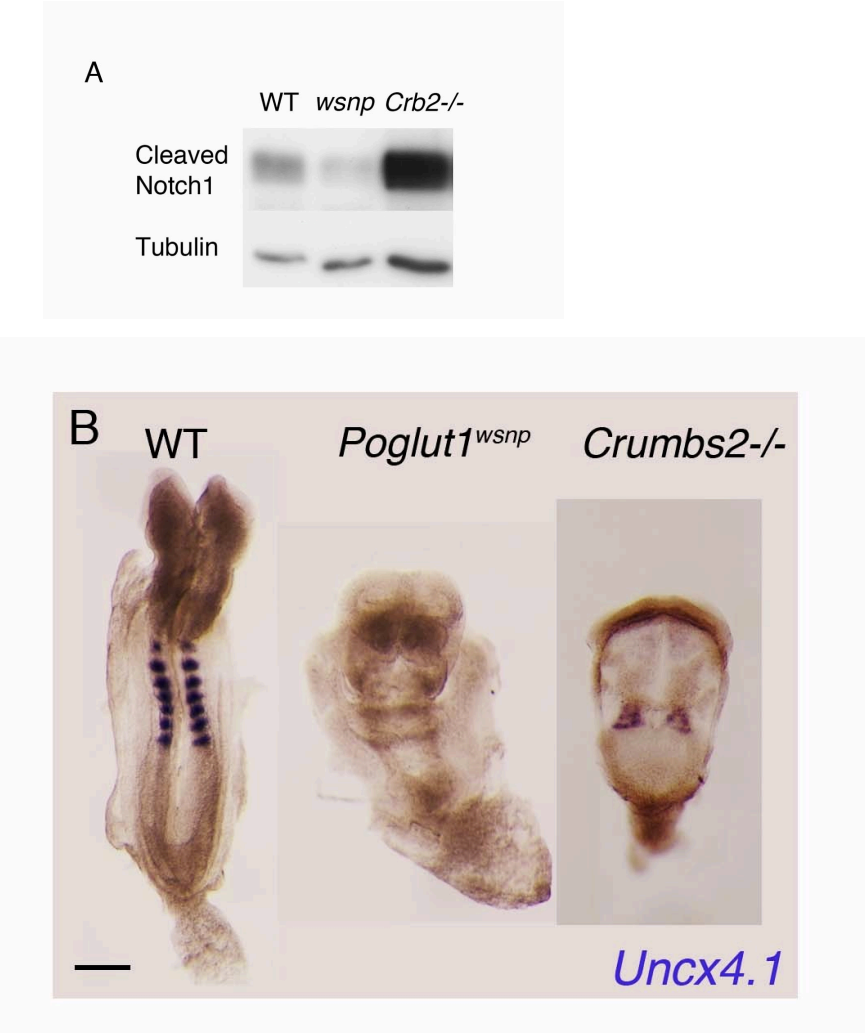


Figure 3.11 Somitogenesis in *Poglut1*^{wsnp} and *Crumbs2* mutants
Western blot analysis of wild type, *Poglut1*^{wsnp} and Crumbs2 embryo lysates at E8.5 probed with Cleaved Notch1 antibody (A). While Cleaved Notch1 levels were reduced in *Poglut1*^{wsnp} mutants, the levels were unaltered in *Crumbs2* mutants. In situ for expression of *Uncx4.1* at E8.5 (B). Its expression is completely absent in *Poglut1*^{wsnp} mutants (n=3), while it is always expressed in *Crumbs2* mutants. Dorsal view with anterior on top. Scale bar – 150 μm.

3.7 Discussion

Crumbs2 has a large extracellular domain with 15 EGF like repeats of which 8 of them can be potentially modified by the addition of O-glucose. It remains to be determined whether the modification of all the 8 EGF repeats or a combination of some EGF repeats (Rana et al., 2011) is essential for its function. The latter could be particularly interesting as it could serve as an added layer of control, which could be regulated in a tissue or stage specific manner. *Drosophila* Notch was modified at all the EGF repeats containing the predicted site. However, the stoichiometry of the modification was cell-type dependent (Rana et al., 2011).

This is the first study that shows the presence of sugar modifications on full-length protein purified from a biological system. Most studies express short peptides containing a few EGF repeats in cell lines and perform mass spectrometry analysis. While short peptides are useful to determine whether a certain EGF repeat can be modified, they do not establish whether the sugar modification exists on the full-length protein in its biological context.

Analysis in *Drosophila* and mammalian cell lines indicate that O-glucose is covalently linked to the serine of the predicted motif (C1-X-S-X-A/P-C2) in EGF repeats. This is extended to give the trisaccharide Xyl-α 1,3-Xyl-α1,3-Glc on all the EGF repeats examined for O-glucosylation (Rana et al., 2011). Likewise, we found this trisaccharide on the extracellular domain of Crumbs2. The precise function of this trisaccharide remains unknown. Fringe-catalyzed fucose modifications on Notch ECD alter ligand specificity in a tissue dependent manner (Okajima et al., 2003; Panin et al., 1997). Although ligands for Crumbs remain to be determined, recent evidence suggests that the Crumbs extracellular domain can bind to Notch

extracellular domain and regulate Notch signaling (Ohata et al., 2011). We did not see any change in Notch signaling in *Crumbs2* mutants suggesting that this interaction probably is not essential during mouse gastrulation. Studies in *Drosophila* and zebrafish suggest that homophilic interactions between the extracellular domains of Crumbs function as a positive feedback mechanism to maintain the apical enrichment of Crumbs (Fletcher et al., 2012; Letizia et al., 2013; Roper, 2012; Zou et al., 2012). Absence of glycosylation of the EGF repeats of Crumbs, could alter these homophilic interactions and therefore cause a reduction in apical enrichment of Crumbs2 in *Poglut1*^{wsnp} mutants. Alternatively, the loss of O-glucose modification could lead to improper protein folding in the endoplasmic reticulum, which then fails to be localized properly in the epithelium. *Rumi* mutants had a mild reduction in the surface localization of Notch (Acar et al., 2008). However, flies with a knock-in allele of *Notch* mutated at all Poglut1 sites did not have any reduction in surface localization (Leonardi et al., 2011). Our observations show that the extracellular domain is important for the membrane localization of Crumbs2, which in turn is important for its function.

In *Drosophila*, both over-expression and loss of Crumbs have adverse phenotypes, indicating that function of Crumbs is sensitive to its level of expression (Tepass, 1996; Wodarz et al., 1995). The recycling and trafficking machinery tightly regulate Crumbs apical membrane localization. *Drosophila* mutants that alter trafficking, such as *Retromer*, affect membrane localization of Crumbs and therefore resemble *Crumbs* mutants (Fletcher et al., 2012; Pocha et al., 2011). Our data suggest that this dependence of Crumbs2 function on its membrane localization is conserved in mammals.

In addition to O-glucose modification, Crumbs2 has 6 potential O-fucosylation sites, but *Pofut1* mutants do not have an apparent Crumbs2 loss of function phenotype (Okamura and Saga, 2008). It remains to be determined whether Crumbs2 is modified with O-fucose and whether Crumbs2 membrane localization is more sensitive to O-glucosylation than other sugar modifications in mammals. Sugar modification on the extracellular domain of Crumbs is dispensable for its function in *Drosophila*. Flies with a knock-in allele of *Crumbs* with mutated Poglut1 target sites were viable and did not have morphological abnormalities (Haltom et al., 2014). Thus the function of Poglut1 in regulating apical membrane localization of Crumbs2 is specific to mammals.

Crumbs1 also has EGF repeats that can be modified. Since we did not observe any change in its expression and *Crumbs1* null mice did not have a gastrulation phenotype, we propose that Crumbs1 does not have an essential role during gastrulation, although we have no data on the role of O-glucosylation of Crumbs1 on its functions later in development. In contrast, the drastic reduction in Crumbs2 expression in *Poglut1*^{wsnp} mutants suggests that this modification is essential for its function.

While we show here that Crumbs2 is the main target of Poglut1 during mammalian gastrulation, we cannot rule out the possibility that, similar to Notch, there are other proteins that are modified by Poglut1 at this stage. However, they seem to have no detectable effect on the phenotype at gastrulation. The function of Poglut1 in modifying other proteins might be more important at later stages of development or in a tissue specific manner.

Chapter 4 Crumbs proteins in mammalian gastrulation

Introduction

The loss of Crumbs2 causes embryonic lethality with a syndrome of defects including improper gastrulation and somitogenesis. At E8.5 while the paraxial and axial mesoderm are specified, they are highly reduced in the mutants as seen by *Meox1* and *Brachyury* expression (Figure 3.7). Mesoderm arises from an organized EMT at the primitive streak in the embryos. Sections through the streak show accumulation of cells at the streak in both *Crumbs2* and *Poglut1*^{wsnp} mutants (Figure 3.8). The *Crumbs2* gastrulation phenotypes were previously attributed to defects in epiblast polarity (Xiao et al., 2011). In this chapter, I will describe the roles of Crumbs2 in gastrulation EMT, which I show are independent of a role in epithelial polarity.

4.1 Expression of Crumbs2 and its complex members during gastrulation

To help understand the role of Crumbs proteins in gastrulation, I first looked at their expression. All three genes are expressed during gastrulation from E6.5 to E8.5 as seen by RT-PCR analysis (Figure 4.1)

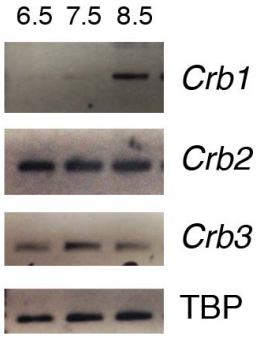


Figure 4.1 Expression of Crumbs genes during gastrulation
RT-PCR analysis for expression of *Crumbs1*, *Crumbs2* and *Crumbs3* transcripts from cDNA synthesized from embryos at embryonic day 6.5, 7.5 and 8.5. All three

Crumbs were expressed at these stages. TATA-Box Binding protein (TBP) was used as a loading control.

Crumbs1 protein localized in the Golgi at E7.5 (Figure 3.4). I then probed for the expression of Crumbs2 during gastrulation. By in situ hybridization I found that *Crumbs2* was expressed in the epiblast cells and its transcript was enriched apically. This apical enrichment of *Crumbs* RNA has also been observed in *Drosophila* (Li et al., 2008). Additionally, there was a gradient of *Crumbs2* expression within the epiblast, with the highest expression at the primitive streak (Figure 4.2B and C). *Crumbs2* was not detectable in the endoderm and mesoderm cells. In the mid-streak stage embryo I found that, similar to its RNA localization, Crumbs2 protein was expressed throughout the epiblast and its intensity was always highest at the primitive streak and it was absent in the endoderm. It localized to the membrane apical to E-cadherin, an adherens junction protein (Figure 4.2D, D'). Crumbs2 partially colocalized with the apical F-actin and extended further apical to it (Figure 4.2F,F'). We could not find a suitable antibody specific for Crumbs3 to observe its localization during gastrulation. However, immunostaining with the pan-Crumbs antibody, which recognizes all three mammalian Crumbs showed uniform distribution in the epiblast of both wild type and *Crumbs2* mutants. This suggests that Crumbs3 was expressed uniformly throughout the epiblast.

As development proceeds, the embryo initiates the formation of neural epithelium and it begins to fold into a tube. At head fold stages, Crumbs2 protein is expressed in the neural epithelium and it continues to be expressed in the epiblast and primitive streak. The posterior-to-anterior gradient of its expression is more apparent in these stages (Figure 4.2E).

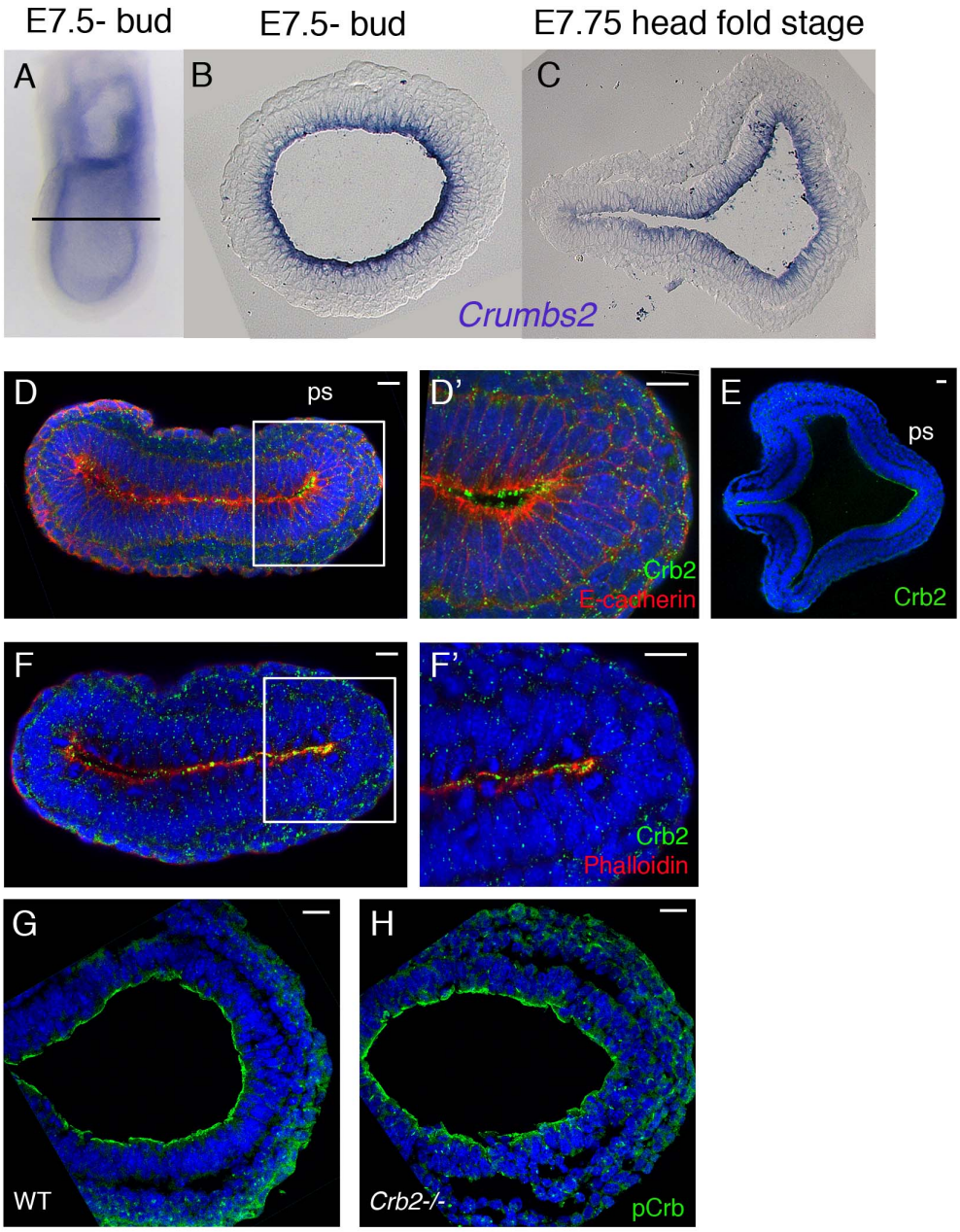


Figure 4.2 Expression and localization of Crumbs2 during gastrulation
Whole mount in situ hybridization for expression of *Crumbs2 (A).* It is expressed only in the epiblast and is not detectable in the endoderm. B and C sections of whole-mount in situ hybridization showing apical enrichment of *Crb2* RNA in epiblast cells and a gradient with highest expression at the primitive streak. D,D',E,F and F' single optical sections from whole mount immunostaining for Crumbs2 and E-cadherin (D.D') and Phalloidin (F,F'). The protein is also expressed in a gradient with highest expression at the primitive streak. (D' and F' are higher magnifications of D and F). At head fold stages, Crumbs2 is also

expressed in the neural epithelium (E). Transverse sections through wild type (G) and *Crumbs2* mutant (H) immunostained with pan-Crumbs antibody showing uniform expression in the epiblast. The uniform pan-Crb expression in *Crb2* mutant suggests that this is the expression pattern of Crb3 as Crb1 is expressed in the Golgi. Notice the Golgi staining for Crb1 in G and H.
All sections: anterior to left and posterior to right. Scale bar -21 μm

To investigate its apical enrichment at the streak, I looked at en face expression of Crumbs2. Unlike other polarity proteins like ZO1 (a tight junction protein), I found strong puncta of Crumbs2 in cells at the streak and non-uniform membrane localization in the epiblast cells at the streak (Figure 4.3). To determine the shape of the cells expressing the strong puncta of Crumbs2, I utilized mice carrying X-linked GFP transgene to outline the shape of the cells. This transgene expresses cytoplasmic GFP in a mosaic manner as a result of X-inactivation in female embryos (Hadjantonakis et al., 2001). I found that the strong Crb2 puncta correspond to the apical membranes of highly constricted cells at the primitive streak (Figure 4.3). I speculate that these constricted cells are at an intermediate step in EMT and are poised to delaminate soon. As expected, I did not see any membrane localization of Crumbs2 in the *Poglut1*^{wsnp} mutant primitive streak.

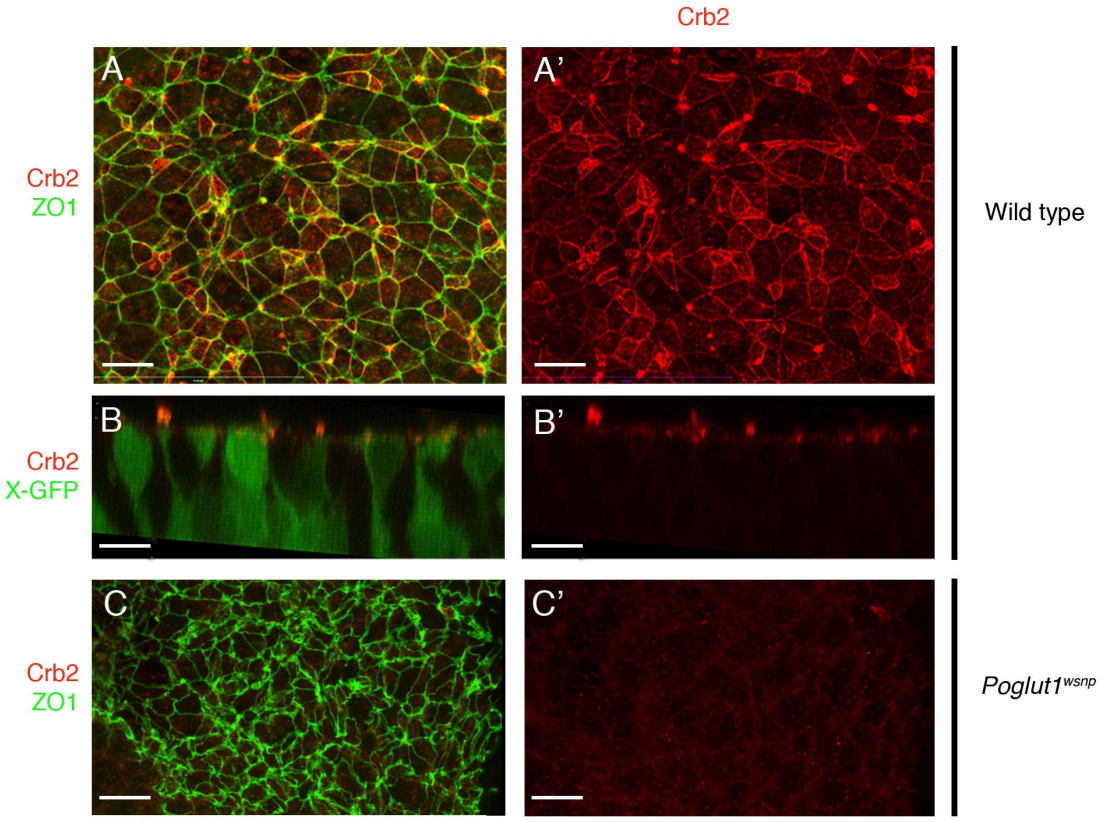


Figure 4.3 En face localization of Crumbs2 at the primitive streak
Single optical sections from whole mount immunostaining for Crumbs2 and ZO1 in wild type (A,A') and *Poglut1*^{wsnp} (C,C') at the primitive streak at E8.0. Unlike the uniform distribution of ZO1, Crumbs2 is not uniform in expression, with some cells showing high expression. The membrane localization of Crumbs2 is lost in *Poglut1*^{wsnp} mutants. 3D reconstruction from whole mount immunostaining for Crumbs2 on wild type embryos at E8.0 with X-linked GFP transgene showing constricted cells with high apical Crumbs2 accumulation (B, B'). Scale bar - 10 μm

I also looked at the expression of Crumbs complex member PatJ. Similar to Crumbs2, PatJ had a non-uniform localization in the primitive streak (Figure 4.4A, B). It was also expressed in the nuclei of a small percentage of dividing cells. Its localization was lost from the apical membrane in both *Crumbs2* and

Poglut1^{wsnp} mutant primitive streaks (Figure 4.4A). This suggests that, similar to results observed in *Drosophila*, the mammalian Crumbs complex also functions together as a unit to maintain membrane expression of one another.

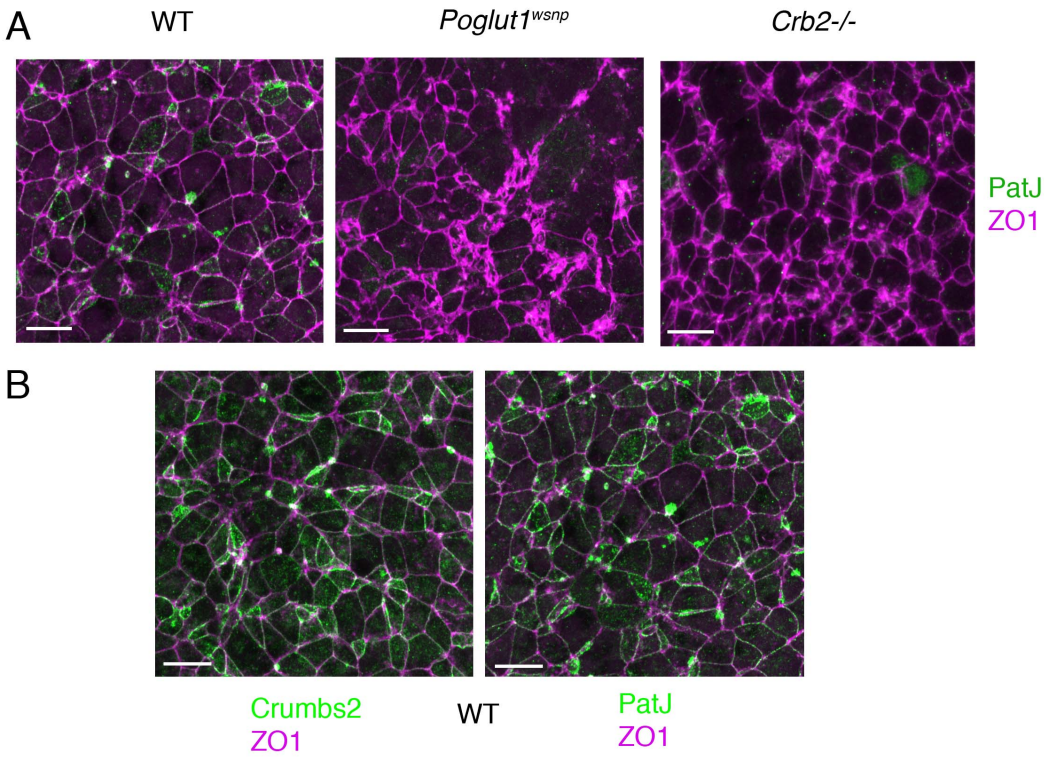


Figure 4.4 PatJ localization at the primitive streak
Immunostaining for en face expression of PatJ and ZO1 (A) in wild type, *Poglut1*^{wsnp} and *Crumbs2* mutants. Its membrane localization is lost in the mutants. (B) Comparison of en face expression of Crumbs2 and PatJ from different embryos, showing similar distributions at the primitive streak at E8.0. Scale bar - 10 μm

The expression pattern of Crumbs2 suggested that it was required in the epiblast rather than the endoderm or extra-embryonic tissues. To test this, I generated epiblast-specific *Crumbs2* mutants by combining the *Sox2-Cre* transgene (Hayashi et al., 2002) with the conditional allele of *Crumbs2*. The phenotypes of these mutants were indistinguishable from the *Crumbs2* mutants. *Meox1* expression shows reduction in paraxial mesoderm and *Brachyury* expression shows discontinuous midline. Similar to *Crumbs2* mutants, they also initiate the somitogenesis clock, as seen by expression of *Uncx4.1* (Figure 4.5).

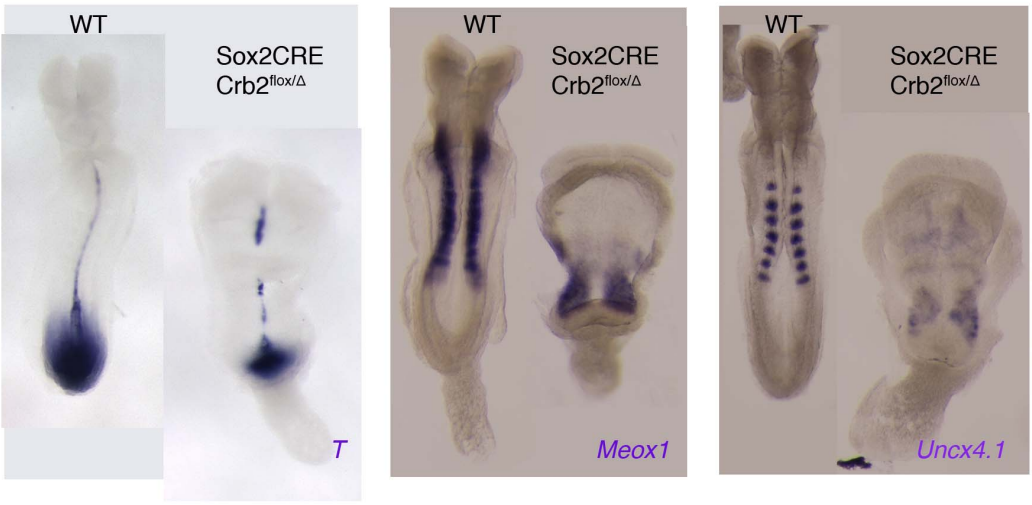


Figure 4.5 Mesoderm defects in epiblast specific deletion of *Crumbs2*
In situ for expression of *Brachyury, Meox1 and Uncx4.1* at E8.5. *Brachyury* expression shows discontinuous midline in the mutants. *Meox1* expression shows reduction in paraxial mesoderm. *Uncx4.1* expression shows that somitogenesis clock is active, however the mutants do not form proper somites like wild type at E8.5.

4.2 Crumbs proteins are not essential for establishment of epiblast polarity

Crumbs2 mutants, although histologically normal at E7.5, had a broader streak compared to wild type by E8 (Figure 3.8). Despite gastrulation beginning at E6.5, the *Crumbs2* mutant embryos do not have a severe phenotype until a day into gastrulation. This could be due to redundancy among the Crumbs family members during gastrulation. To investigate the potential role of other members of the Crumbs family in this process, I generated double and triple null mutant embryos. *Crumbs1*$^{rd8/rd8}$ *Crumbs2-/-* double mutant embryos morphologically resemble *Crumbs2* mutants suggesting that Crumbs1 does not play an essential role during gastrulation (Figure 3.4). I generated mice lacking *Crumbs2* and *Crumbs3*. *Crb2-/-*, *Crb3-/-* double mutant embryos phenotypically resembled *Crb2-/-* single mutants, as seen by in situ for *Meox1* expression at E8.5 (Figure 4.6).

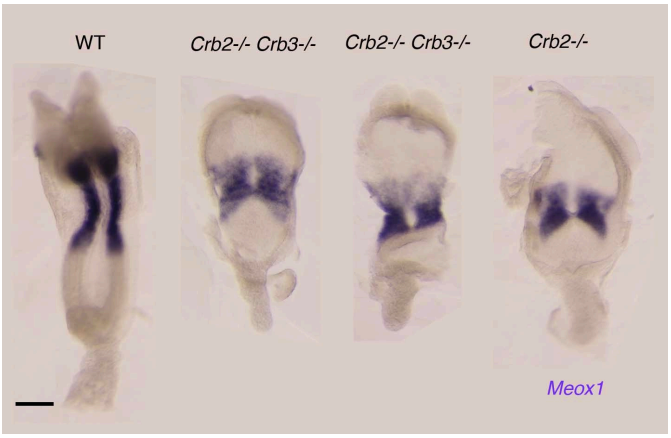


Figure 4.6 Analysis of *Crumb2-/- Crumbs3-/-* double mutants.
In situ for expression of *Meox1* in wild type, *Crumbs2* mutant and *Crumbs2 Crumbs3* double mutants at E8.5. The double mutants look indistinguishable from *Crumbs2* mutants. Scale bar- 150 μm

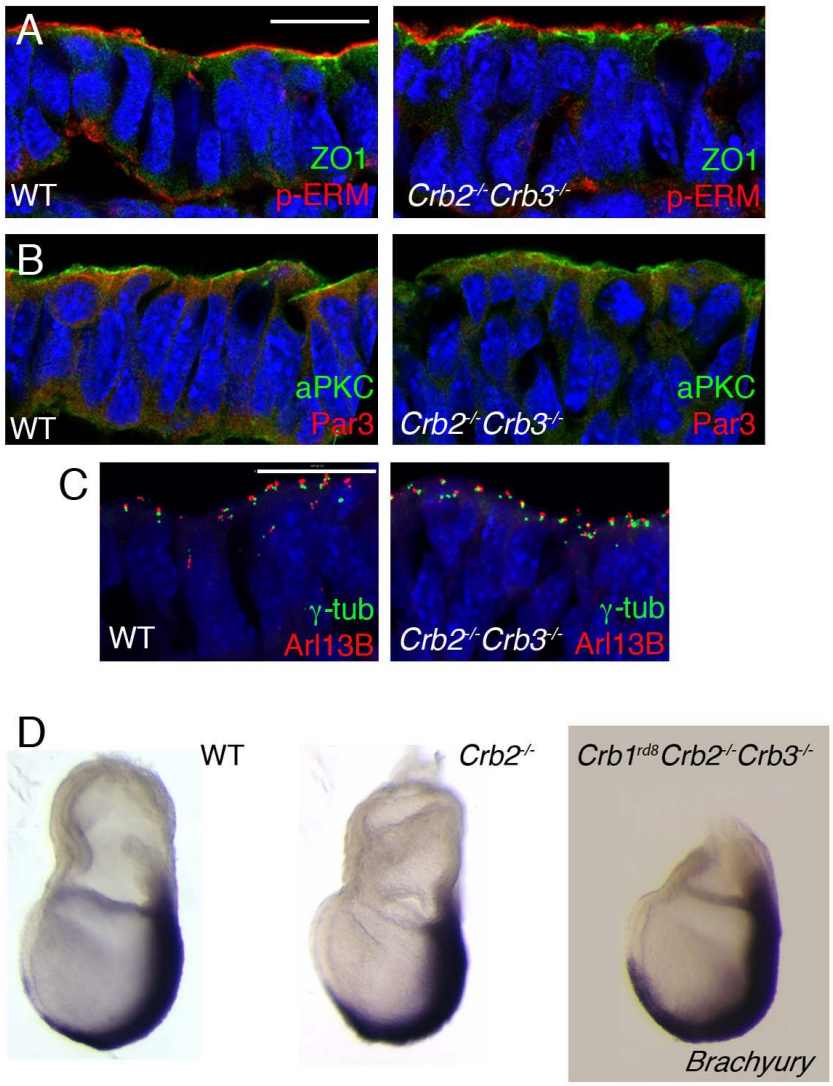


Figure 4.7 Crumbs proteins are not essential for establishment of epiblast polarity

Transverse sections through the epiblast of E7.75 wild type and *Crb2-/- Crb3-/-* double mutant embryo immunostained for ZO1 and pERM (A), aPKC and Par3 (B) and γ-tubulin and Arl13b (C). The double mutants did not have defects in epiblast polarity. In situ for expression of *Brachyury* in wild type, *Crumbs2* mutants and Crumbs triple mutants. Besides the broader domain of *Brachyury* expression, the triple mutants did not have a more severe gastrulation phenotype than the *Crb2* single mutants. The extra-embryonic portion of the triple mutant was removed for genotyping. Scale bar - 21 μm

To investigate the role of Crumbs proteins in establishing epithelial polarity, I examined the *Crb2-/- Crb3-/-* double mutants for apical-basal polarity. The localization of ZO1 a component of the tight junction and pERM present in the apical membrane of the cells were maintained in the mutants (Figure 4.7A). The Par complex (Par3 and aPKC) localizes to the apical region of the cells and their localization was not perturbed in the mutants (Figure 4.7B). The apical-basal polarity of the epiblast was not affected in either the *Crumbs2-/-* mutants or the double mutants. An isoform of Crumbs3 was implicated in primary ciliogenesis and its loss led to absence of cilia in cell lines (Fan et al., 2007). I investigated the presence of primary cilia in double mutants with Arl13b, which marks the ciliary membrane and γ-tubulin, which labels the centrosomes (Caspary et al., 2007). I did not observe any loss of primary cilia in the mutants (Figure 4.7C). I generated triple null embryos that lack all known members of the Crumbs family. I found that the phenotype of the triple mutants is similar to that of *Crumbs2* mutants at E7.5 (Figure 4.7D). The triple mutants did not have a more severe gastrulation phenotype such as *Fgf8* knockout (Sun et al., 1999) or polarity phenotype like *E-cadherin* (Riethmacher et al., 1995). Similar to *Crumbs2* mutants, they had a broader streak as seen by the expansion of *Brachyury* expression (Figure 4.7D). This indicates that Crumbs proteins are not essential for the establishment of epiblast polarity, that gastrulation is the first function of Crumbs family in mammalian development and this process is regulated primarily by Crumbs2.

4.3 Crumbs2 is required for the gastrulation EMT

The earliest defect observed in the *Crumbs2* mutants was the increase in the width of the primitive streak as seen by *Brachyury* expression (Figure 4.7) (Xiao et al., 2011). At early bud stage (E7.5), about a day into gastrulation, I saw that

both wild type and *Crumbs2* mutants have primitive streaks of similar width, seen both by laminin breakdown and Brachyury expression (Figure 4.8A, B, Figure 3.10). However by E8.0, I saw an increase in the width of the primitive streak indicating an accumulation of cells expressing E-cadherin (Figure 3.10).

The accumulation of cells at the streak could be due to either a defect in EMT or a failure of the mesoderm cells to migrate following EMT. To determine which process is affected, I looked at the expression of Sox2, an epiblast marker, and Snail1, a mesoderm marker. In wild type embryos, Sox2 is expressed in the epiblast and down regulated at the primitive streak, while Snail1 is up-regulated as cells undergo EMT at the streak (Figure 4.8C, E). These cell layers were cleanly separated in the wild type. As early as E7.5, when the streak width was similar to wild-type, I found some mutant cells at the streak whose nuclei were further away from the apical side of the epiblast still expressed Sox2 (asterisks, Figure 4.8D). This defect was exacerbated at E8.0, when I saw more Sox2 positive cells accumulated at the streak, pointing to a defect in EMT rather than cell migration (Figure 4.8F). The mutant mesoderm cell layer consisted of Snail1 positive cells indicating that the cells could undergo EMT and migrate away. I observed a similar thickening of the epiblast and predominantly Sox2 positive cells at the streak in *Poglut1*^{wsnp} mutants (Figure 4.8G). In both *Crumbs2* and *Poglut1*^{wsnp} mutants I noticed Snail1 positive cells scattered amidst Sox2 expressing cells in the streak both at E7.5 and E8.5, suggesting that while cells could undergo EMT, they were probably unable to segregate and be incorporated into mesoderm layer efficiently. The accumulation of cells in *Crumbs2* and *Poglut1*^{wsnp} mutants was different from that observed in mutants for cell migration following EMT such as *Rac1* mutants (Migeotte et al., 2011). Unlike *Crumbs2* or *Poglut1*^{wsnp}

mutants, *epiblast-deleted Rac1* mutants had an accumulation of Snail1 positive cells at the primitive streak. This suggests that the defect in *Crumbs2* and *Poglut1*^{wsnp} was in gastrulation EMT rather than cell migration.

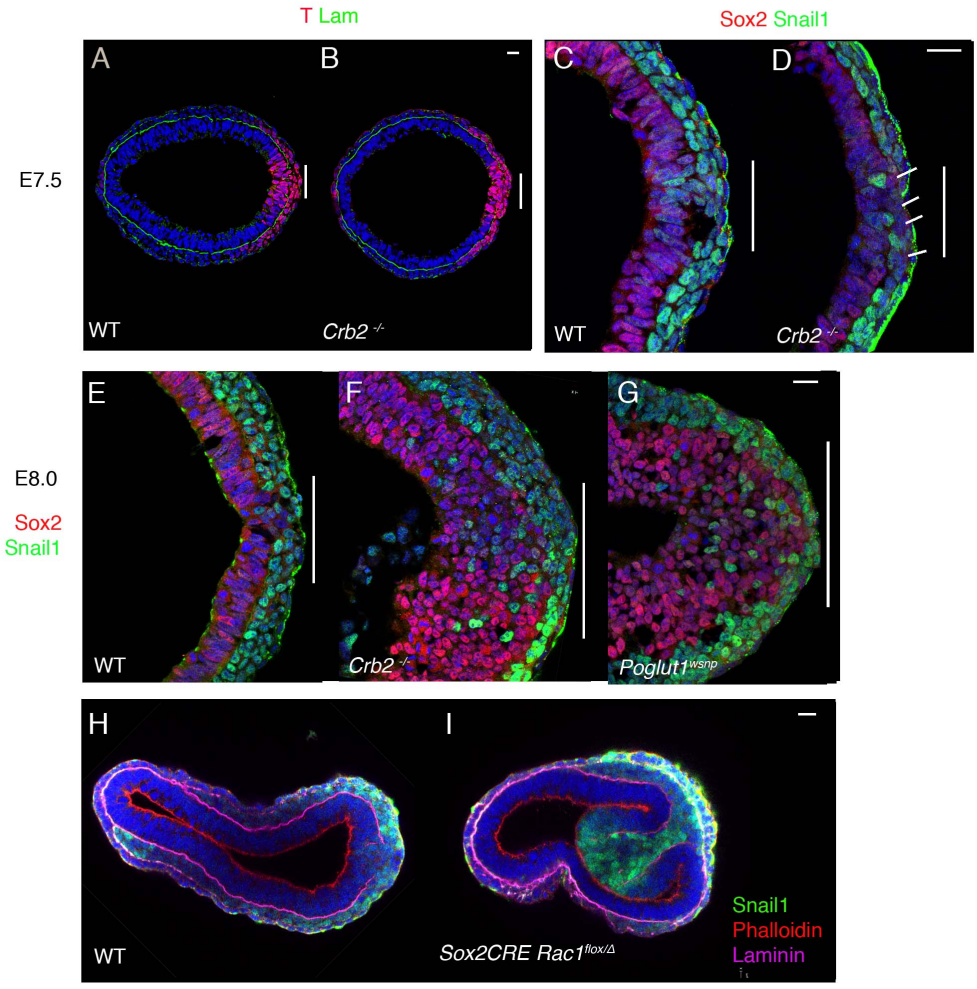


Figure 4.8 Crumbs2 is required for gastrulation EMT
Transverse sections through primitive streak at E7.5 (A,B,C,D) and E8.0 (E,F,G) immunostained for Brachyury (T) and Laminin (A,B) and Snail1 and Sox2 (C,D,E,F,G). Bars indicate width of primitive streak. Even when the streak sizes are almost similar, cells begin expressing Sox2 being to accumulate at the streak (arrows D). By E8.0, this is amplified with the thickening of the epiblast. Nascent mesoderm cells do express Snail1. Notice the scattered Snail1 positive cells at the streak between Sox2 positive cells (F, G). Single optical sections from whole mount immunostaining for Snail1, Laminin and Phalloidin in wild type (H) and

embryo with epiblast specific deletion of *Rac1* (I). Notice the Snail1 positive cells accumulated at the primitive streak in epiblast-deleted *Rac1* mutants. Scale bar - 21 μm.

4.4 *Crumbs2* mutant primitive streak cells fail to detach from the apical surface of the epiblast

Since most of the cells accumulated at the primitive streak in *Crumbs2* mutants expressed Sox2, we wanted to determine whether they were still epithelial. I used the scanning electron microscopy (SEM) to observe the shape of the cells. A cross section through the primitive streak of wild type embryo at E7.5 shows epiblast cells delaminating to give rise to mesoderm cells (Figure 4.9A,A'). In *Crumbs2*-/- mutants, I saw elongated epithelial cells with thin projections still attached to the apical surface of the epiblast (Figure 4.9B, B'). I observed similar highly constricted cells maintaining apical attachments in the primitive streak of *Poglut1*^{wsnp} embryos (Figure 4.9C, C').

To help visualize the cells at the streak at a cellular resolution, I utilized mice carrying X-linked GFP transgene, which has a mosaic pattern of expression in the female embryos and can be used to outline single cells (Hadjantonakis et al., 2001). Wild-type embryos had constricted cells ready to delaminate only at the streak and cells further away from the streak did not maintain any connection with the apical surface of the epiblast (Figure 4.9D). In contrast, cells further away from the streak still maintained attachment to the apical surface of the epiblast (Figure 4.9E, F) in the mutants. These attachments still had adherens junctions as seen by E-cadherin expression (Figure 4.9H). This suggested that although the cells had initiated EMT by losing their basement membrane and

changing cell shape, they were unable to down-regulate E-cadherin and delaminate from the streak**.** Therefore they were unable to successfully complete EMT. It is interesting to note that in en face views of the wild-type streak, Crumbs2 was enriched in the apical membrane of highly constricted cells that would potentially delaminate at the primitive streak (Figure 4.3). This suggested that Crumbs2 enrichment was required for efficient cell delamination during gastrulation EMT.

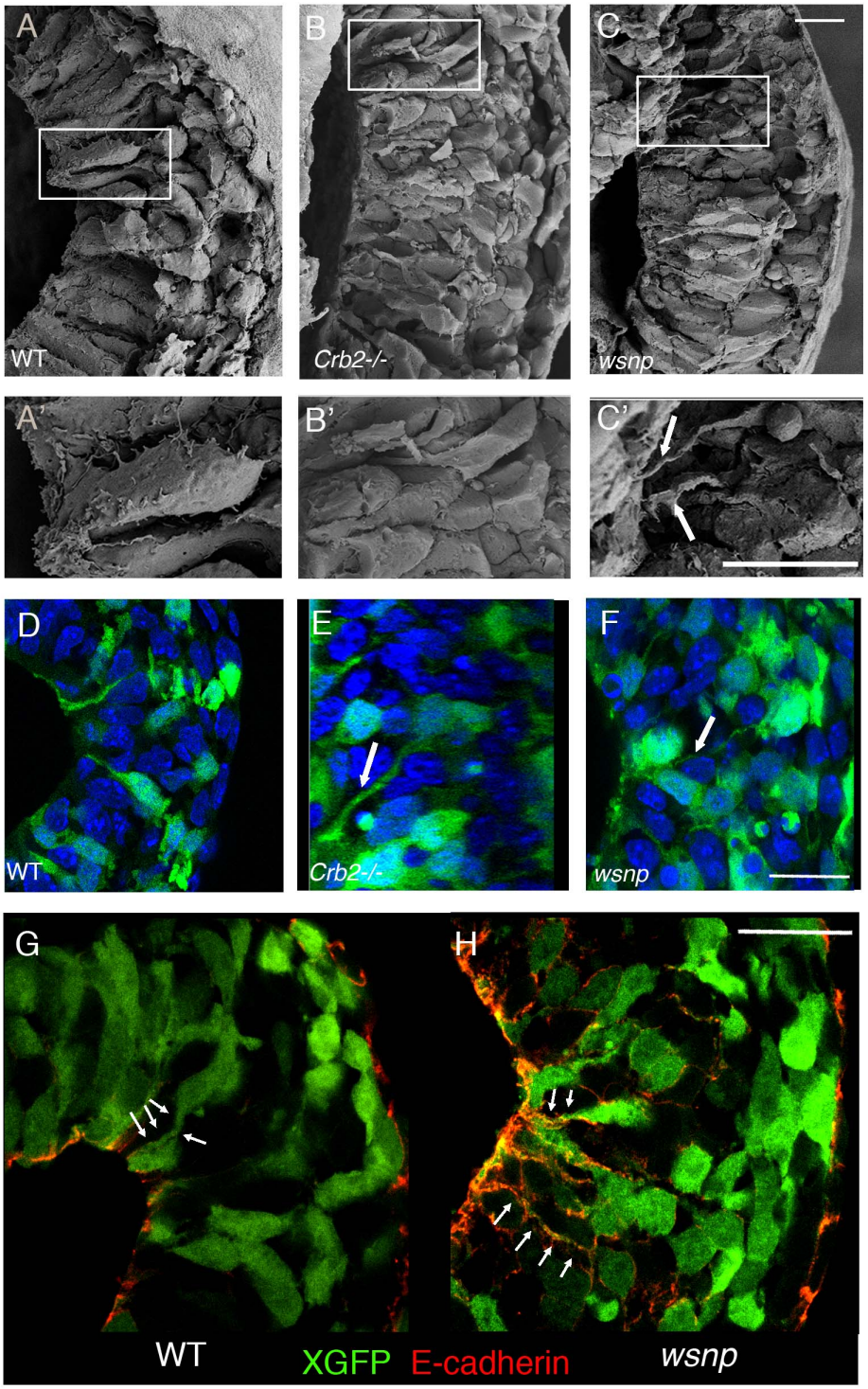


Figure 4.9 Crumbs2 is essential for cell delamination at the primitive streak Scanning electron microscope views of primitive streak in wild type (A,A'), *Crumbs2* (B,B') and *Poglut1*^{wsnp} (C,C') mutants. Arrows point to highly constricted cells at the primitive streak of the mutants compared to the wild type. A', B' and

C' are higher magnification of the primitive streak showing constricted cells in the mutants. Single optical section from 3-D reconstruction of transverse sections through the primitive streak of wild type (D), *Crumbs2* (E) and *Poglut1*^{wsnp} (F) embryos expressing X-linked GFP in a mosaic manner. Arrows show cells with their base (nuclei) away from the streak still maintaining their apical attachment, indicating a failure to delaminate. Single optical sections of transverse sections throught the primitive streak of wild type (G) and Poglut1wsnp mutants (H) expressing X-linked GFP in a mosaic manner immunostained for E-cadherin (red). Arrows point to constricted cells in (G) and (H). Notice the presence of E-cadherin in constricted cells in the mutant (H). Scale bar – A-C'-10 μm, D-H- 24 μm.

4.5 Crumbs2 is required for the organization of the neural epithelium

In addition to the EMT defect at the streak, I observed that the mutant epiblast and neural epithelium were thicker than in wild-type embryos. Using Sox2 as a marker for neural epithelium, I measured the thickness of the neural epithelium in wild-type and mutant embryos. I found that the neural epithelium was ~1.4 times thicker in the *Crumbs2* mutants than in wild type. In addition to having a thicker epithelium, the nuclei were more round and more randomly oriented (Figure 4.10G'', H''). The long axis of the nuclei in the neural epithelium of wild type was oriented along the apical–basal axis of the neural epithelium (Figure 4.10G''). However, in the mutants, the long axis of the nuclei was not aligned to the apical-basal axis of the epithelium (Figure 4.10H''). Laminin marks the basement membrane of the neural epithelium, and the mesoderm layer is sandwiched between the two basement membrane layers in wild type. The relative lack of mesoderm cells was apparent in the mutant, as there were lesser cells between the two basement membrane layers (Figure 4.10A,D). In addition, while wild type embryos have a continuous thick basement membrane, we start to see breaks in the basement membrane of mutant neural epithelium (Figure 4.10 D, H'). Cells expressing Sox2 extend beyond these breaks in the basement

membrane (Figure 4.10H), suggesting that the epithelial integrity has been compromised. This could eventually lead to the cell mixing observed by Xiao et al, 2011.

I also found that there was a considerable reduction in the apical cell surface area in the *Crumbs2* mutants compared to wild type. ZO1 marks the tight junctions and was apically localized in the neural epithelium. Using ZO1 to mark cell boundaries, I measured the apical cell surface area in wild types and mutants. I found that the cell surface area was reduced in the mutants (Figure 4.10B,E). To observe the shape of the cells in the mutants, I used the mosaic X-linked GFP transgenic mice. I found that the cells of the neural epithelium look morphologically different in the *Crumbs2* mutants. The cells were more apically constricted and elongated basolaterally compared to those in the wild-type epithelium (Figure 4.10C,F). This corresponded to a reduced apical domain and an expansion of basolateral domain, a characteristic of loss of Crumbs in *Drosophila* (Richard et al., 2009). Despite these changes in cell shape, the polarity of the epithelium was maintained.

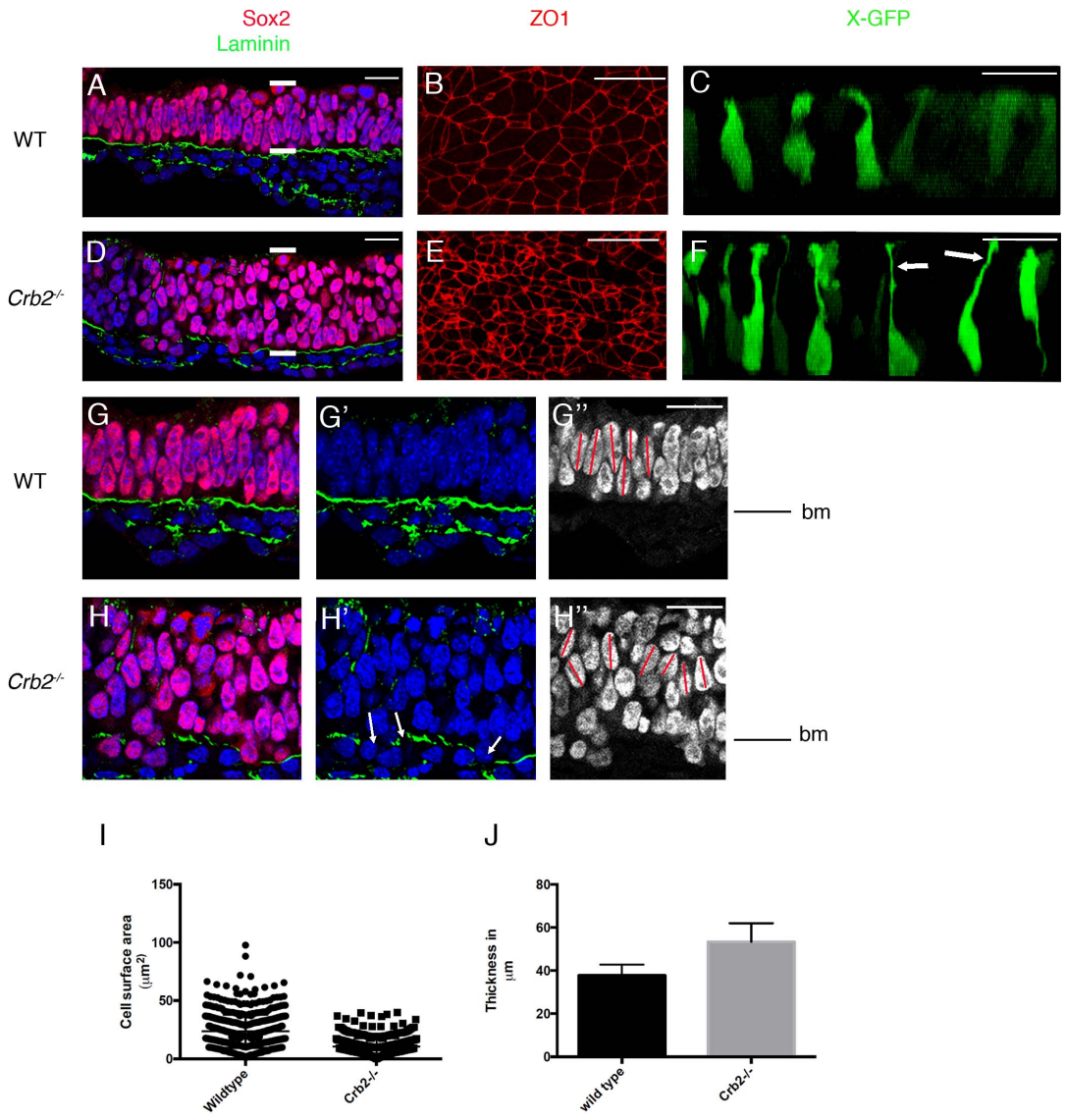


Figure 4.10 Crumbs2 is required to maintain epithelial integrity
Single optical section of transverse section through the neural epithelium of wild type (A) and *Crumbs2* (D) mutants. Sox2 and laminin staining show the increased thickness of the neural epithelium in the mutants at E8.0.
En face view of the neural epithelium of wild type (B) and *Crumbs2*$^{-/-}$ (E) stained with ZO1. This shows the reduction in the apical surface area of the cells in the *Crumbs2* mutants. 3-D reconstruction of cells in the neural epithelium of wild type (C) and *Crumbs2*$^{-/-}$ (F) embryos expressing X-linked GFP. The cells in the mutants have reduced apical surface area and an increased basolateral domain.

(G) and (H) higher magnification of (A) and (D). Mutant neural epithelium has breaks in laminin expression and Sox2 positive nuclei extend beyond these breaks (H,H'). Sox2 positive nuclei never extend beyond the basement membrane in the wild type (G,G'). (G'') shows the long axis of the nuclei (in red) aligned along the apical-basal axis of the epithelium. (H'') shows long axis of the nuclei (in red) randomly oriented.
(I) Quantification of cell surface area (avg ± SEM), WT = 23.64±0.60 and *Crb2*$^{-/-}$ = 10.50±0.34, P<0.0001
(J) Quantification of neural epithelium thickness (avg ± SEM), WT = 37.75±0.92 and *Crb2*$^{-/-}$=53.25±1.60, P<0.0001.
bm-basement membrane. Scale bar -21 μm.

4.6 Hippo signaling appears to be normal in mouse *Crumbs2* mutant embryos

Drosophila Crumbs has been implicated in regulating the Hippo signaling cascade via its interaction with Expanded, a FERM domain protein. However, a role for Crumbs proteins in mammalian Hippo signal transduction remains to be investigated. It is noteworthy that mice lacking *YAP* or *Tead1* and *Tead2* have a gastrulation phenotype (Sawada et al., 2008). I looked at the ratio of phosphorylated to total YAP from whole embryo extracts. I did not see any difference between the ratio in wild type and *Crumbs2* mutants at E8.5 (Figure 4.11) suggesting that Hippo signaling might not be perturbed in these embryos. However, it might be worth investigating the sub-cellular localization of YAP and p-YAP as these could be affected.

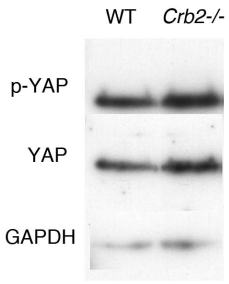


Figure 4.11 Hippo signaling in *Crumbs2* mutants
Western blot analysis of wild type and *Crumbs2* whole embryo lysates probed with YAP, pYAP and GAPDH antibody.

4.7 Cell proliferation and cell death are not affected in *Crumbs2* mutants

The thickening of the epiblast and the neural epithelium led us to investigate the rate of proliferation in the *Crumbs2* mutants. Sections were stained for phospho-histone H3, a marker for mitotic cells and I calculated the mitotic index in wild type and *Crumbs2* mutant neural epithelium. The mitotic indexes were not significantly different (WT = 9.924 ± 0.9685 (avg ± SEM) and *Crumbs2* mutant = 7.002 ± 0.5897 (avg ± SEM), p value = 0.02 not significant), although the percentage of non-apical mitosis was significantly higher in the mutants. (Non-apical mitosis in wild type = 2.6% and *Crumbs2* mutants = 23.5%, Figure 4.12). To investigate cell death, sections were stained for cleaved-caspase, an apoptotic marker. I did not see any significant differences between the wild type and mutants. This suggests that the increase in thickness of the epithelia is not a consequence of changes in cell proliferation or cell death.

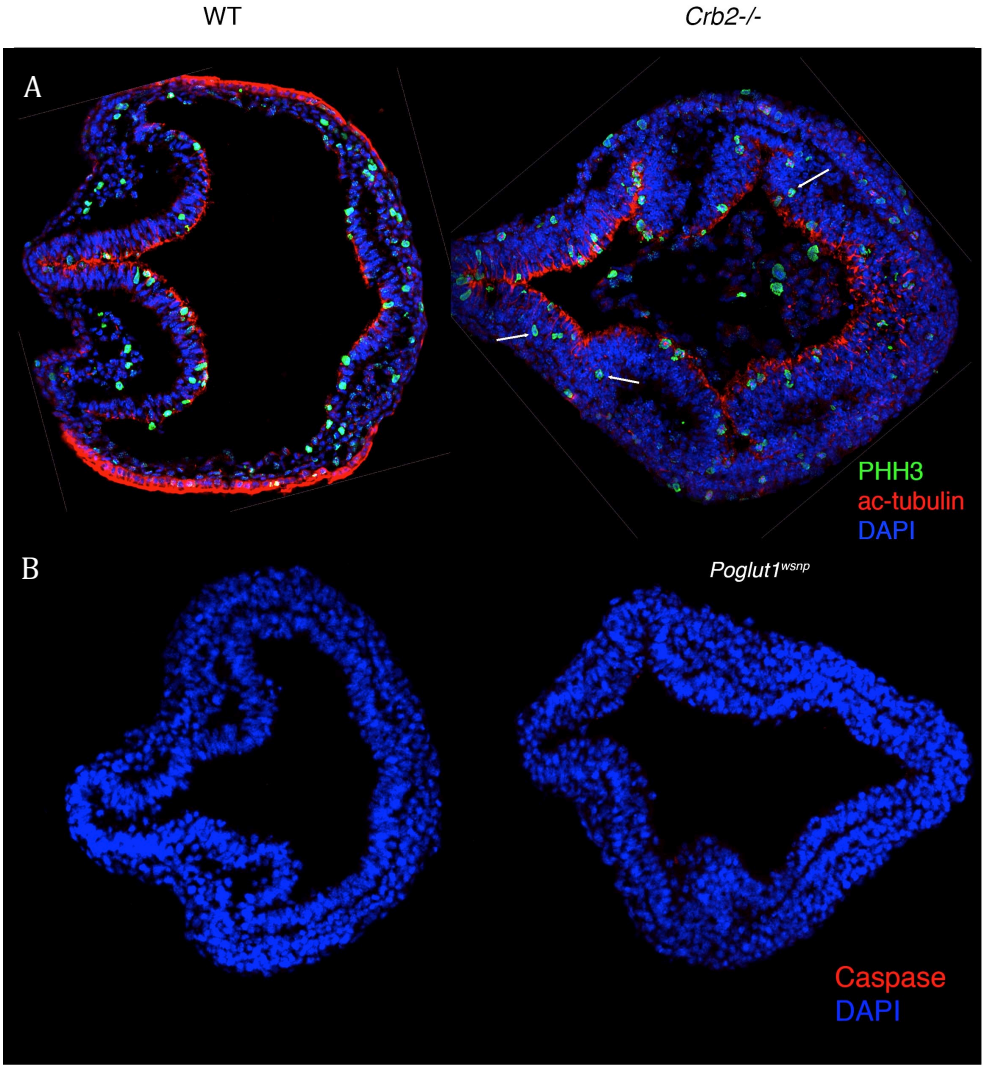


Figure 4.12 Mitotic index and cell death in Crumbs2 mutants
Transverse sections through the wild type, Crumbs2 and *Poglut1*^{wsnp} embryos at E8.0 immunostained for pHH3 (mitotic cells), acetylated-tubulin (A) and caspase (B). Mitotic index calculated as (total pHH3 positive nuclei/total DAPI nuclei) in neural epithelium. Anterior is on the left. Arrows point to examples of non-apical pHH3 positive nuclei.

4.8 Crumbs2 is required for epiblast integrity only when the primitive streak is present

The epithelia in the *Crumbs2* mutant were highly disorganized at E7.75. Both the epiblast and the neural epithelium were considerably thicker than the corresponding wild type. At the primitive streak, the cells undergo an organized EMT and delaminate from the epiblast epithelium. We hypothesized that the streak was more sensitive to the loss of Crumbs2 as the streak was a dynamic epithelium with cells delaminating and acquiring new neighbors constantly, and the epiblast might require Crumbs2 to reinforce polarity in this dynamic epithelium. To test this, we decided to prevent primitive streak formation in the *Crumbs2* mutants. Mouse *Wnt3* mutants are unable to form a primitive streak and therefore proliferate as a bag of epiblast cells. A cross section through the *Wnt3* mutants (Figure 4.13B) revealed two cell layers, the epiblast, which uniformly expressed Sox2 and the visceral endoderm (Sox2 negative). These two cell layers were separated by a continuous basement membrane. I generated *Crumbs2 Wnt3* double mutants and analyzed the epiblast epithelium. Morphologically, the double mutants looked similar to *Wnt3* single mutants (Figure 4.13A, C). A cross-section through the embryos stained for E-cadherin and laminin expression showed that, even at a cellular level, the double mutants resembled the *Wnt3* single mutants (Figure 4.13B, B', D, D'). Since the embryos were folded, we could not measure the apical surface area of the epiblast cells. Unlike the *Crumbs2* mutants (Figure 4.10H), I did not observe any breaks in the basement membrane as seen by continuous laminin expression in both *Wnt3* and *Crumbs2 Wnt3* double mutants (Figure 4.13B', D'). In addition, I do not see any Sox2 positive nuclei extending beyond the basement membrane in the *Crumbs2 Wnt3* double mutants (Figure 4.13F). This suggests that the loss of epithelial integrity in

Crumbs2 mutants could be rescued *Crumbs2 Wnt3* double mutants. This indicated that the absence of the primitive streak in the *Crumbs2 Wnt3* mutants prevented the epithelial disorganization defect I observed in *Crumbs2* single mutants. This suggested that the primitive streak was particularly sensitive to the loss of Crumbs2, and that disruption of the primitive streak affected the rest of the epiblast epithelium and led to the disorganization we observed. I suggest that Crumbs2 is essential for maintaining epithelial integrity only when tissues are undergoing rearrangements, i.e., Crumbs2 is required at the primitive streak to maintain epithelial integrity when cells undergo EMT.

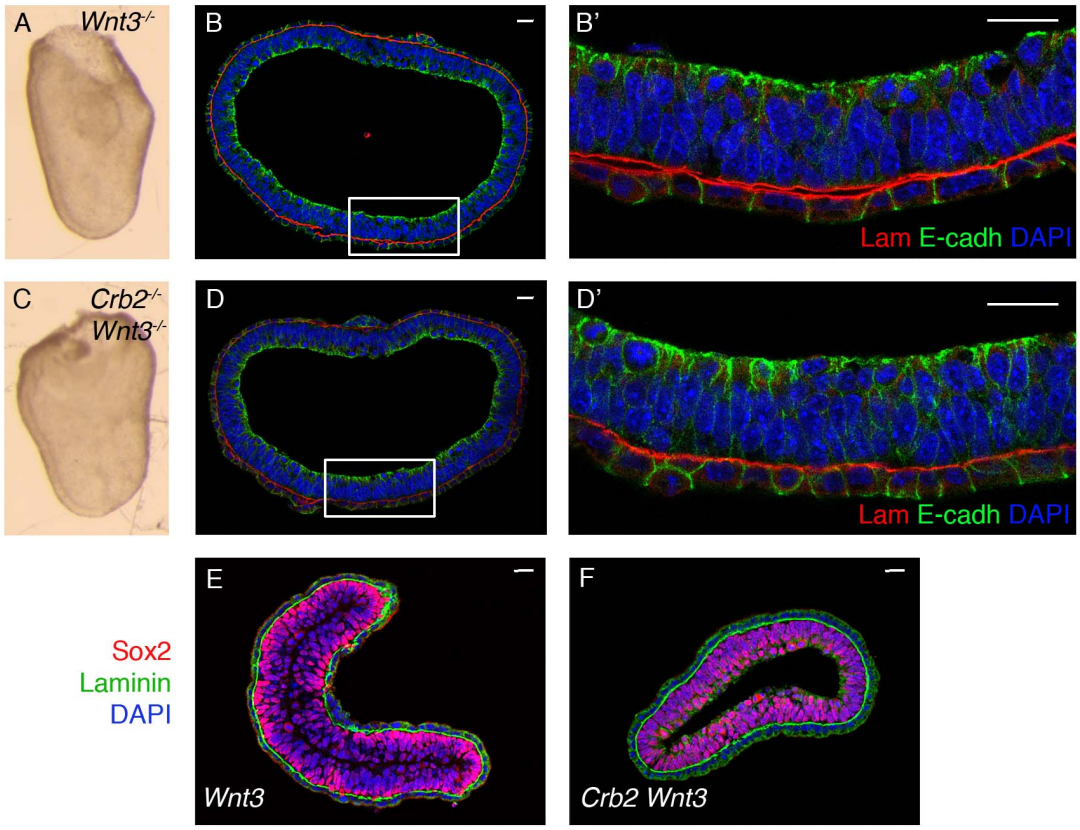


Figure 4.13 Crumbs2 is required for epiblast integrity only when cells delaminate at the primitive streak
Wnt3$^{-/-}$ (A) and *Crumbs2*$^{-/-}$ *Wnt3*$^{-/-}$ (C) double mutant embryos showing the plane of sections in B and D. *Wnt3*$^{-/-}$ mutants do not form a primitive streak and remain as a bag of epithelial cells.
Transverse section through the embryos immuno-stained with Laminin (red) and E-Cadherin (green). Higher magnification of the epithelium of *Wnt3*$^{-/-}$ (B') and *Wnt3*$^{-/-}$ *Crb2*$^{-/-}$double mutants (C') shows that the double mutants do not breaks in laminin expression as seen in *Crb2*$^{-/-}$ single mutants, suggesting that the epithelial integrity is not affected in the double mutants.
Transverse sections through *Wnt3* (E) and *Crb2 Wnt3* (F) double mutants immunostained for Sox2 (red) and Laminin (green). There are no Sox2 positive nuclei extended outside the basement membrane in the double mutants. Scale bar - 21 µm

4.9 Crumbs2 is required for maintaining epiblast integrity at the primitive streak

The expression pattern of Crumbs2 and the data with *Crumbs2 Wnt3* double mutants suggest that Crumbs2 functions primarily at the primitive streak. In an independent test of whether Crb2 function was required only at the primitive streak, I generated embryos with *Crumbs2* deleted specifically at the primitive streak using *Brachyury (T)*-CRE and the conditional allele of *Crumbs2*. CRE expression in this line begins at mid-to-late streak stage. This is reflected in the phenotype, as embryos with *Crumbs2* deleted using Brachyury-CRE make considerably more mesoderm than *Crumbs2* null mutants. The mutants form a midline heart, unlike the *Crumbs2* mutants, where the heart fields fail to fuse to form a single heart tube. However, these embryos showed reduction in mesoderm-derived tissues and therefore failed to elongate along the anterior-posterior axis as much as wild type at E8.5 (Figure 4.14A). This suggests that Crumbs2 function at the primitive streak is important during gastrulation.

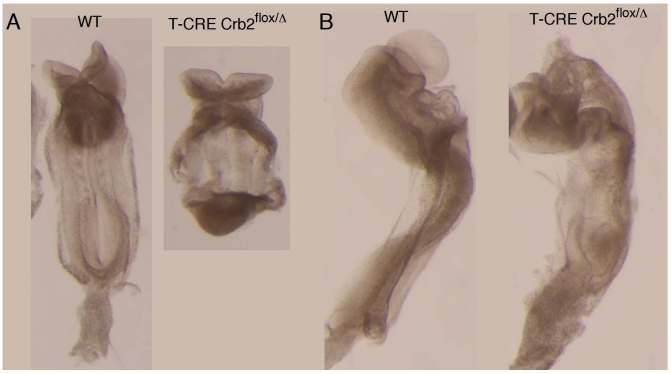


Figure 4.14 *Conditional* deletion of *Crumbs2* in primitive streak
Embryos with Crumbs2 deleted in the late primitive streak recapitulate the deficit of mesoderm phenotype observed in *Crumbs2* null mutants. (A) ventral view and (B) lateral view of two different embryos with anterior up.

4.10 Crumbs2 is required for re-organization of actin-myosin cytoskeleton at the primitive streak

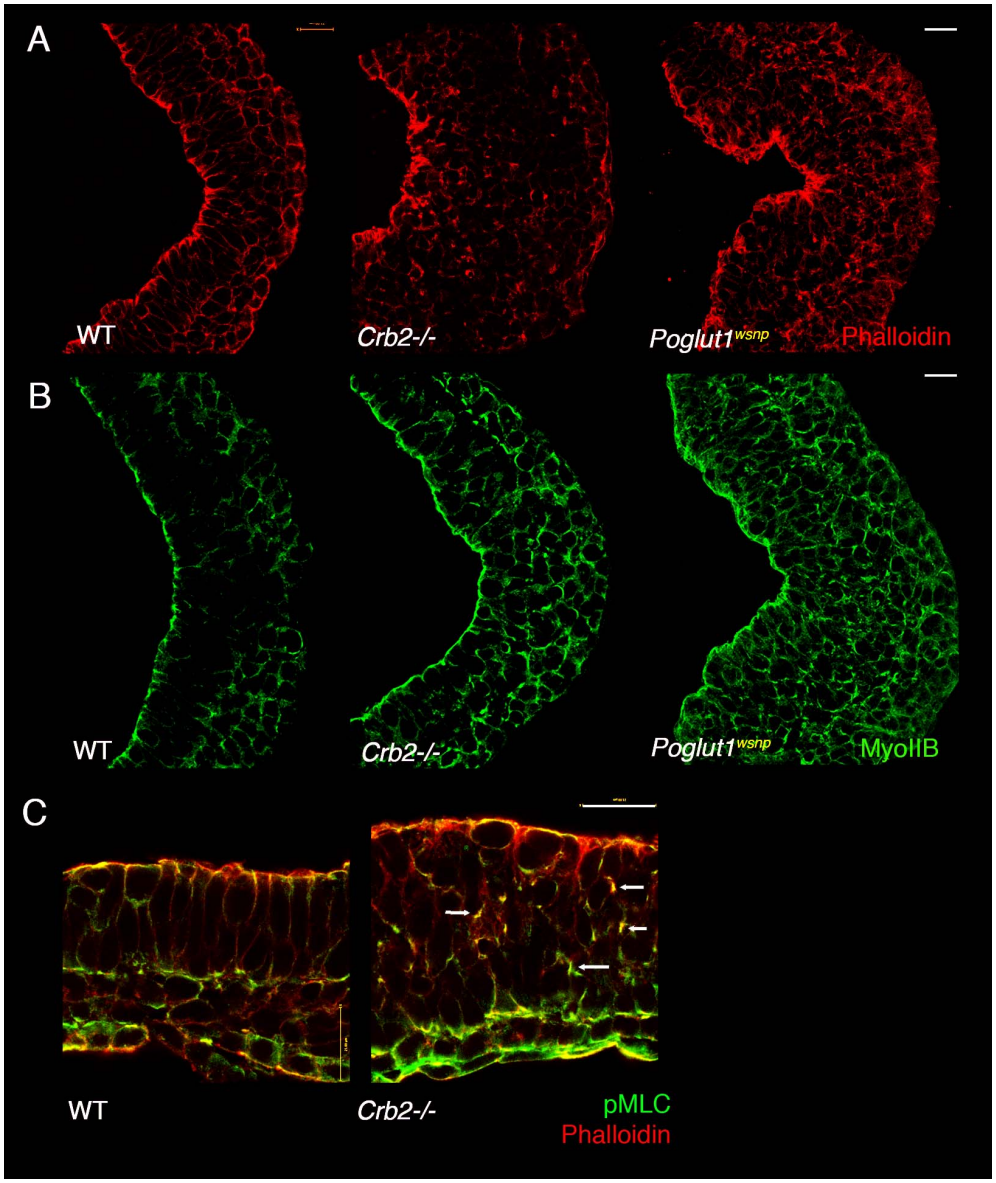


Figure 4.15 Cytoskeletal defects in *Crumbs2* mutants
Transverse sections through the primitive streak of WT, *Crb2*-/- and *Poglut1*^{wsnp} mutants at E7.75 immunostained for Phalloidin (A) Myosin Heavy Chain (B) and phospho-Myosin Light Chain S19 (C). Arrows point to ectopic accumulations of myosin and actin. Scale bar – 21 µm

In *Crumbs2* mutants, we saw an accumulation of cells at the streak that fail to delaminate (Figure 4.8 and 4.9). Cytoskeletal reorganization is an important aspect of EMT. Crumbs has been shown to regulate the cytoskeleton of cells through Moesin and β-heavy-spectrin interacting with its intracellular domain (Letizia et al., 2011; Medina et al., 2002). To determine the role of Crumbs2 in the epiblast cytoskeleton, I looked at the localization of actin and myosin. In wild type embryo, actin and myosin are enriched apically at the streak and epiblast, and they are re-organized upon EMT to facilitate cell migration. In *Crumbs2* mutants, although actin and myosin were apically enriched, I saw ectopic actin and myosin along the cells at the primitive streak (Figure 4.15A, B). I observed the similar ectopic actin and myosin accumulation in the primitive streak of *Poglut1*^{wsnp} mutants. Phosphorylation of the cytoplasmic myosin light chain 2(MLC) on Ser19 is required for actomyosin contractility (Bresnick, 1999). Similar to localization of the Myosin heavy chain, I found ectopic localization of pMLC in *Crumbs2* mutant epiblast (Figure 4.15C).

Anisotropic membrane localization of Crumbs was shown to regulate the organization of myosin cable assembly to facilitate tissue invagination (Roper, 2012). Based on the inverse correlation between Myosin cables and Crumbs membrane localization observed in *Drosophila* salivary placodes, I investigated the apical Myosin network at the primitive streak in *Crumbs2* mutants. There was a general reduction in the myosin at the apical surface of the primitive streak (Figure 4.16A''), while levels of ZO1 were unaffected (Figure 4.16B''). This suggests that Crumbs2 is required to maintain the apical myosin network to facilitate cell delamination.

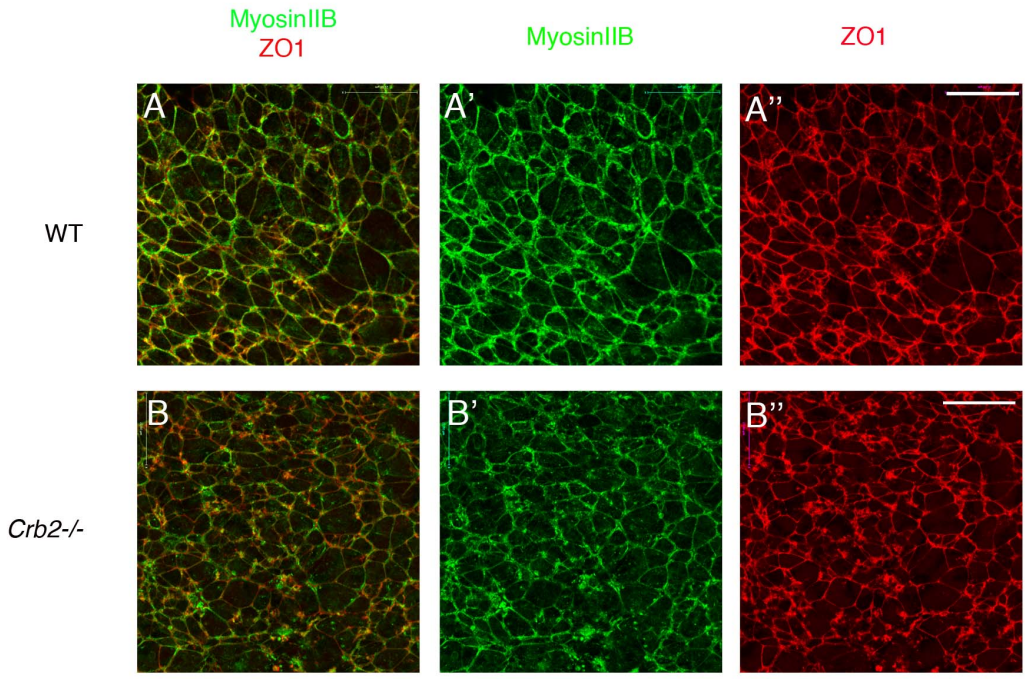


Figure 4.16 En face localization of Myosin Heavy Chain
En face view of primitive streak of wild type (A) and *Crumbs2* mutants (B) immunostained for Myosin Heavy Chain (A', B') and ZO1 (A'', B'') at E8.0. The *Crumbs2* mutants have reduced Myosin heavy chain on their apical surface compared to wild type. The levels of ZO1 remain unchanged (A",B"). Notice the many constricted cells in the mutant primitive streak. Anterior is on top. Scale bar - 21 μm.

4.11 Non-cell autonomous behaviors of *Crumbs2* mutant cells in chimeric embryos

I generated *Crumbs2* mutant ES cells that expressed a GPI-GFP membrane marker to visualize the mutant cells in chimeric embryos. Mutant cells were injected into unlabeled wild type blastocysts. Following blastocyst injections, chimeric embryos were analyzed at E8.5 for the contribution of mutant cells to different lineages (Figure 4.17). Out of total 40 chimeric embryos, I divided them into high mutant cell contribution (n=11) and low mutant cell contribution (n=29).

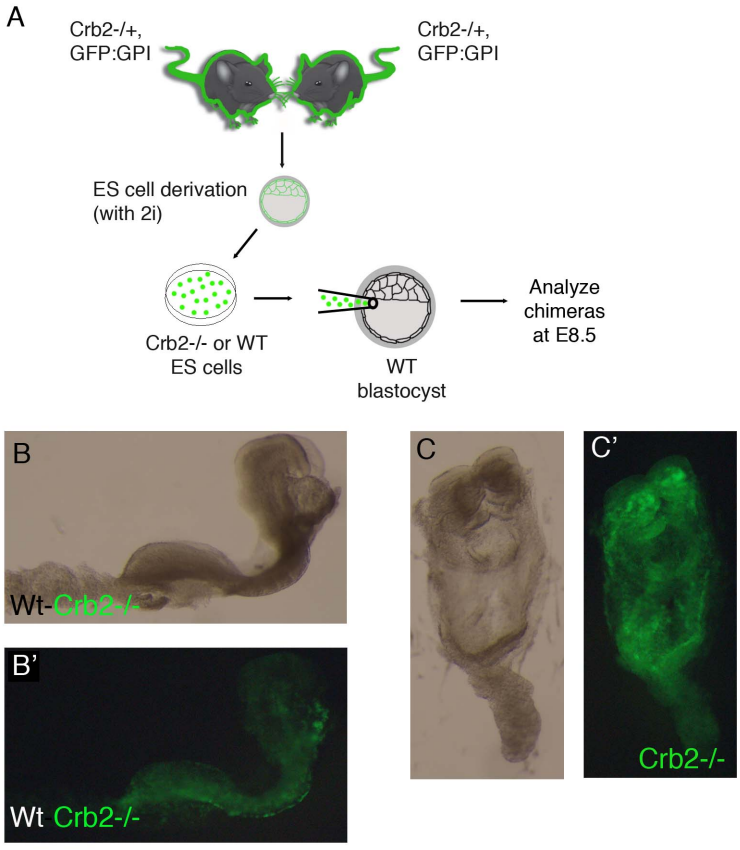


Figure 4.17 Generation of chimera
(A) Schematic showing the generation of mouse chimeras with *Crb2*-/- mutant cells labeled with GPI-GFP. Low contribution chimeras resembled wild type embryos at E8.5 (B, B'). High contribution chimeras showed defects similar to *Crb2* mutants (C, C').

4.11.1. Analysis of high contribution chimeras

High percentage chimeras (95-100% mutants, n=11) recapitulated the *Crumbs2* phenotype (Figure 4.17C, C'); as ES cells do not contribute to extra-embryonic lineages, this indicates that the *Crumbs2* phenotype depends on the phenotype of epiblast cells only, as predicted by the Sox2-Cre phenotype. I scored for percentage of mutant cells in different layer to see if there was any bias in the contribution of mutant cells. In high contribution chimeras, the epiblast was disorganized, similar to *Crumbs2* mutants. I did not observe any bias in the contribution of mutant cells towards the epiblast and neuroepithelial layer or the mesoderm layer (Figure 4.18). There was no accumulation or grouping of either wild-type or mutant cells. Lack of bias of wild-type cells in the mesoderm layer suggests that there is no preferential delamination of wild-type cells in these chimeric embryos.

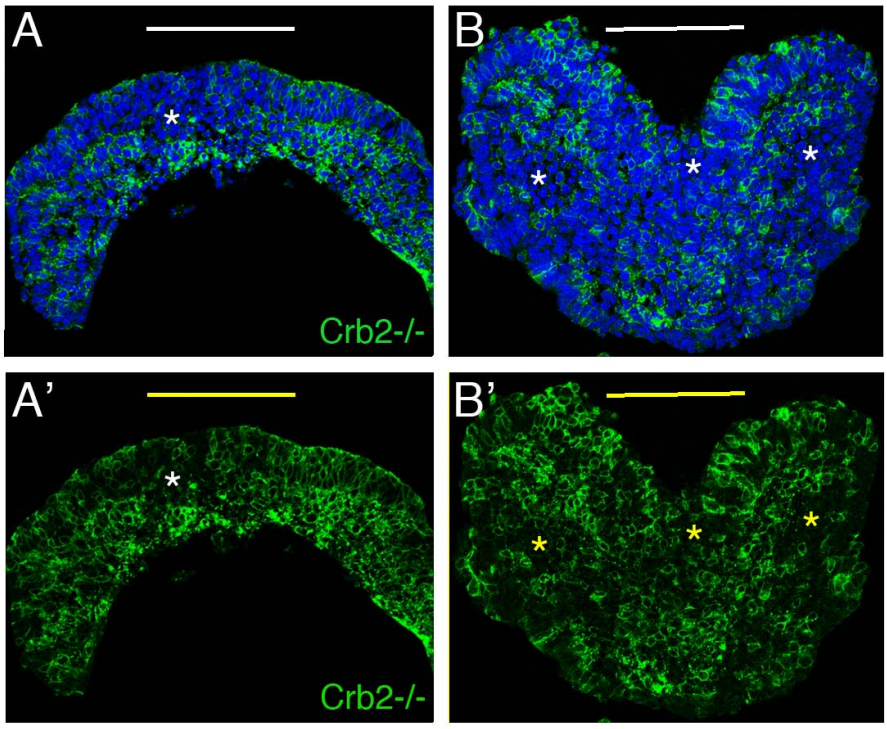


Figure 4.18 Analysis of high contribution chimeras
Transverse sections through the primitive streak of two different high mutant cell contribution chimeric embryos. Mutant cells – green. Bars indicate primitive streak width. The percentages of mutant cells in epiblast and mesoderm layer were scored across a few serial sections. There was no bias in percentage of mutant cells in the epiblast or the nascent mesoderm layer. Asterisks indicate wild type cell groups lacking GFP.

4.11.2. Crumbs2 regulates its localization non-cell autonomously

Drosophila Crumbs can regulate its own localization by trans homophilic interactions between the extracellular domains of Crumbs molecules. To determine whether these homophilic interactions were conserved in mammals, we investigated the localization of Crumbs2 protein in chimeric embryos. Crumbs2 membrane localization was lost in wild type cells neighboring mutant cells in both the primitive streak (Figure 4.19B) and the neural epithelium (Figure 4.19A). However, the localization of actin (Figure 4.19B''') and β-catenin

(Figure 4.19A’’’) were unaltered at these boundaries. This behavior was easier to observe in the neural epithelium compared to the streak as constricted cells at the streak interfered with the analysis. This loss of membrane Crumbs2 localization at the boundaries with wild-type cells in the chimeras could explain the mutant like behavior of wild type cells in high contribution chimeras. Similar non-autonomous regulation of Crumbs localization had been observed in many studies with mosaic *Crb* mutant clones in *Drosophila* (Chen et al., 2010; Hafezi et al., 2012; Pellikka et al., 2002; Roper, 2012). Thus mammalian Crumbs2 can non-cell autonomously stabilize its own membrane localization probably through trans homophilic interactions.

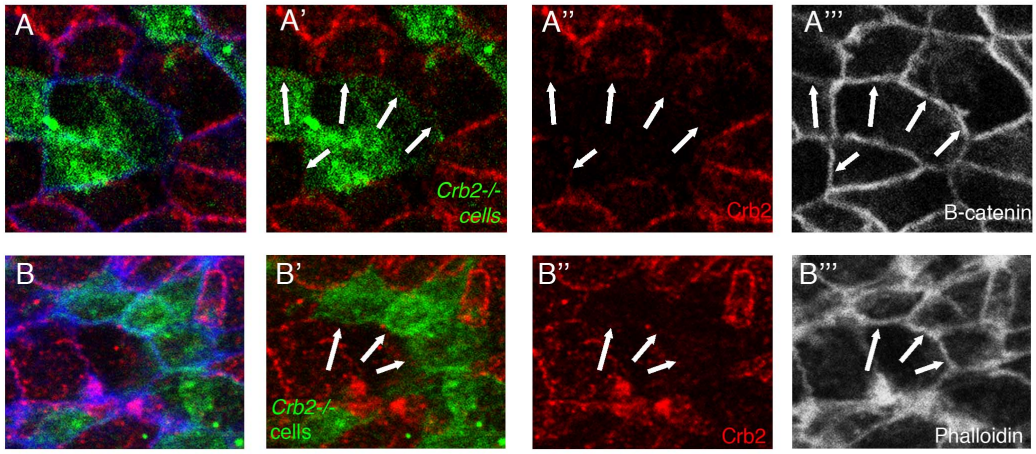


Figure 4.19 Crumbs2 regulates its own localization non-cell autonomously
En face view of the neural epithelium (a) and primitive streak (b) of chimeric embryos at E8.5 immunostained for Crumbs2 (red) and β-catenin (white-A) and Phalloidin (white-B). Mutant cells are GFP positive. Arrows point to the loss of Crumbs2 expression in wild-type cells at the edges shared with mutant cells while β-catenin and phalloidin expression are maintained at these edges.

4.11.3. Low contribution Chimeras

In the presence of high percentage of wild type epiblast cells (n=20 embryos), mutant cells could delaminate from the epiblast and be incorporated into both normal-appearing ectodermal lineage, including the neural epithelium and otic vesicle (Figure 4.20A) and mesodermal lineages including endocardium and myocardium (Figure 4.20B), somites (Figure 4.20C, inset) and gut endoderm (Figure 4.20D). Although *Crumbs2* mutants do not form properly condensed somites like wild type, *Crumbs2* mutant mesoderm cells were incorporated into somites in chimeric embryos, indicating that they had undergone a mesodermal-to-epithelial transition (MET). The mutant cells were present in both the primitive streak and the nascent mesoderm without any bias (Figure 4.20D). Analysis of chimeras with mutant *Fgfr1* cells showed that accumulation of mutant

cells in the epiblast led to ectopic neural tubes (Ciruna et al., 1997). We did not observe any accumulation of mutant or wild type cells in the chimeras. Thus in the presence of a higher percentage of wild-type cells, mutant cells were able to delaminate and behave normally.

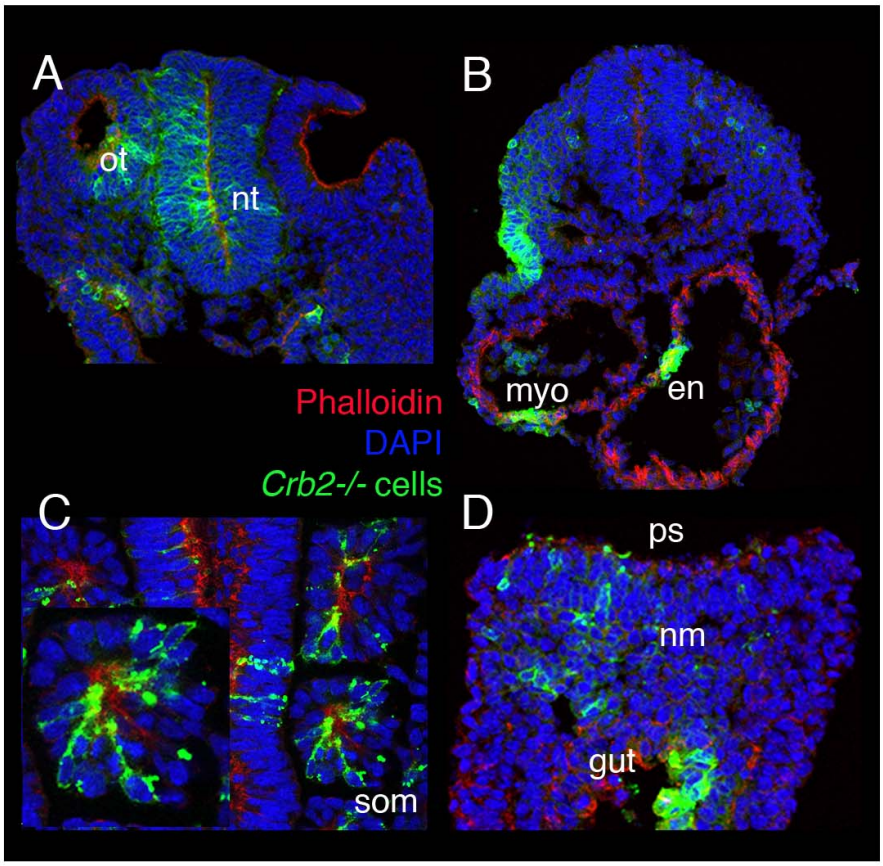


Figure 4.20 Analysis of low-contribution chimeras
Transverse sections through chimeric embryos with moderate contribution of mutant cells immuno-stained with phalloidin (red) showing the contribution of mutant cells (green) to neural epithelium and otic vesicle (A), endocardium and myocardium (B), somites (C), and gut (D). Sections through the primitive streak show equal percentage of mutant cells in both the epiblast and nascent mesoderm indicating that these cells undergo EMT and do not accumulate at the streak (D). Ot-otic vesicle, nt-neural tube, en-endocardium, myo-myocardium, som-somite, ps-primitive streak, nm-nascent mesoderm.

The results from chimera analysis suggest that Crumbs2 primarily functions to facilitate efficient cell delamination at the primitive streak. Once the mutant cells have properly delaminated, they can behave like wild-type cells.

4.12 Live imaging EMT at the primitive streak

In order to determine whether the cells take longer to delaminate in the *Crumbs2* mutants, I imaged cells delaminating at the primitive streak in live mouse embryos from E7.5. To track single cells in the embryo, I labeled cells in a mosaic manner using mice carrying both *mT/mG* and *EllA-CRE* transgenes. Both wild type and *Crumbs2* mutants were imaged in a similar manner with their posterior side facing the objective (Figure 4.21).

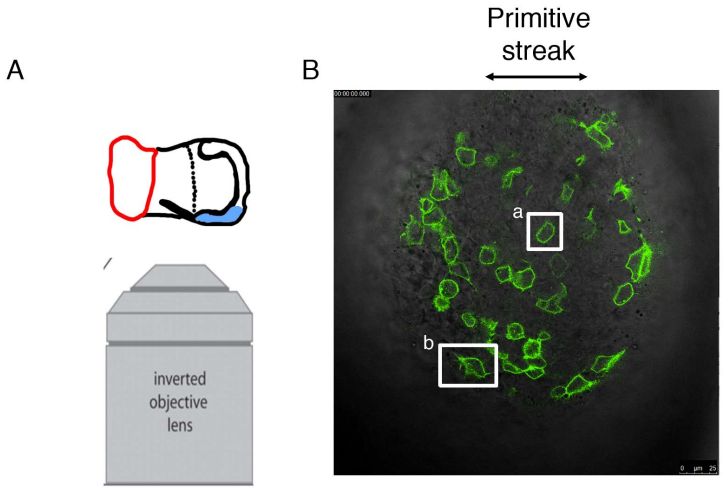


Figure 4.21 Live imaging cell delamination and mesoderm migration
Scheme showing the position of the embryo during live imaging (A). Embryos were places with their primitive streak (posterior side) facing the objective lens. Single optical section showing mosaic GFP labeling of cells at the primitive streak. (a) Shows epithelial cell at the streak and (b) shows nascent mesoderm cell emerging from the primitive streak at E7.5.

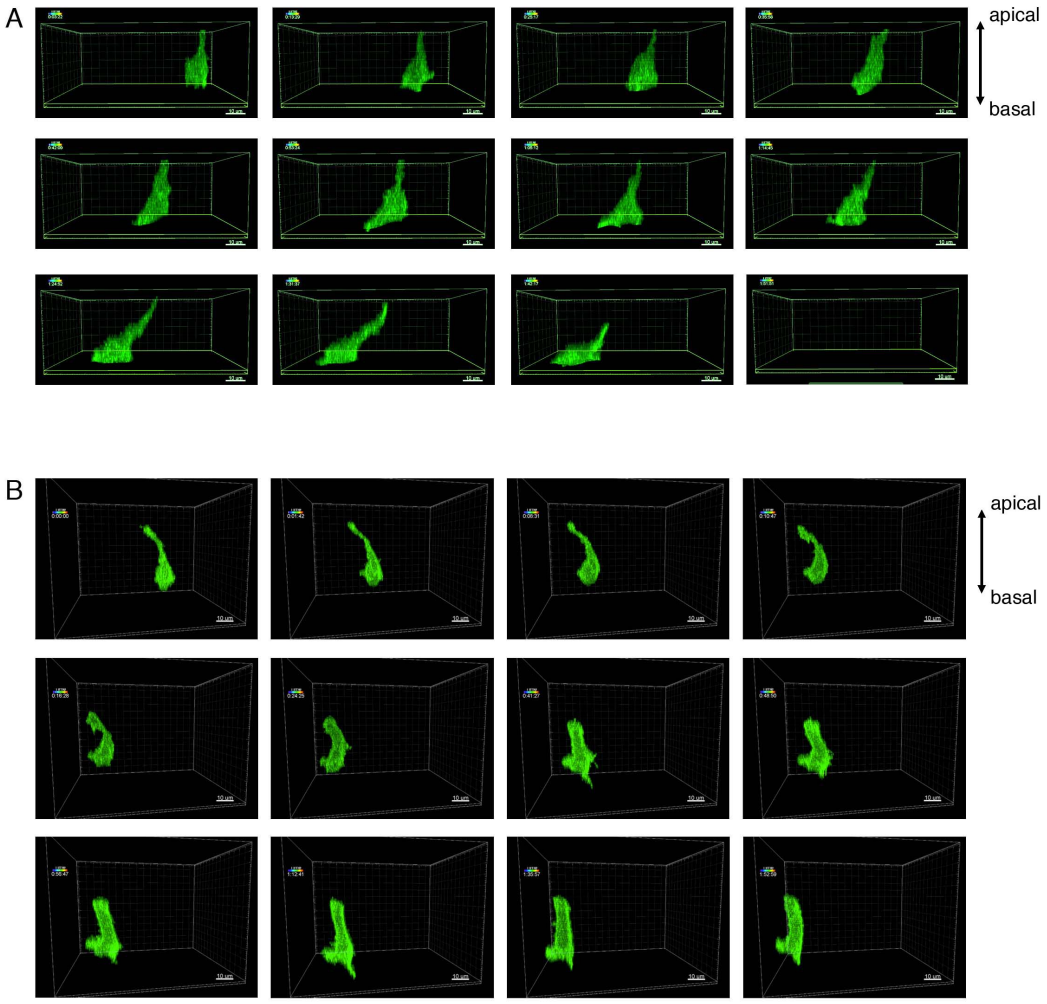


Figure 4.22 Live imaging shows cell delamination is affected in *Crumbs2* mutants Snapshots from time-lapse imaging of wild type (A) and *Crumbs2* (B) mutant embryos showing single cells delaminating at the primitive streak. In wild type embryos (A), the cell constricts its apical membrane and detaches its apical connection to delaminate from the epithelium in less than 2 hours. In *Crumbs2* mutants, the cell constricts its apical membrane and sends out basal protrusions but fails to leave the epithelium (B). Time interval – 10 minutes, scale bar – 10 µm. Apical side of the epithelium is on top.

In wild type embryos the cells are very dynamic. They constrict their apical membrane and leave the epithelium in less than 2 hours (Figure 4.22A). In *Crumbs2* mutants, the cells constrict their apical membrane, but are unable to leave the epithelium (Figure 4.22B). They undo the apical constriction and send out basal protrusions but remain in the epithelium for up to 4 hours. This suggests that cells in the primitive streak of *Crumbs2* mutants begin the process of delamination, but are unable to complete the process or take longer to do so compared to wild type. This indicates that the accumulation of cells in the primitive streak of *Crumbs2* mutants is due to the inability of the cells to delaminate efficiently like wild type.

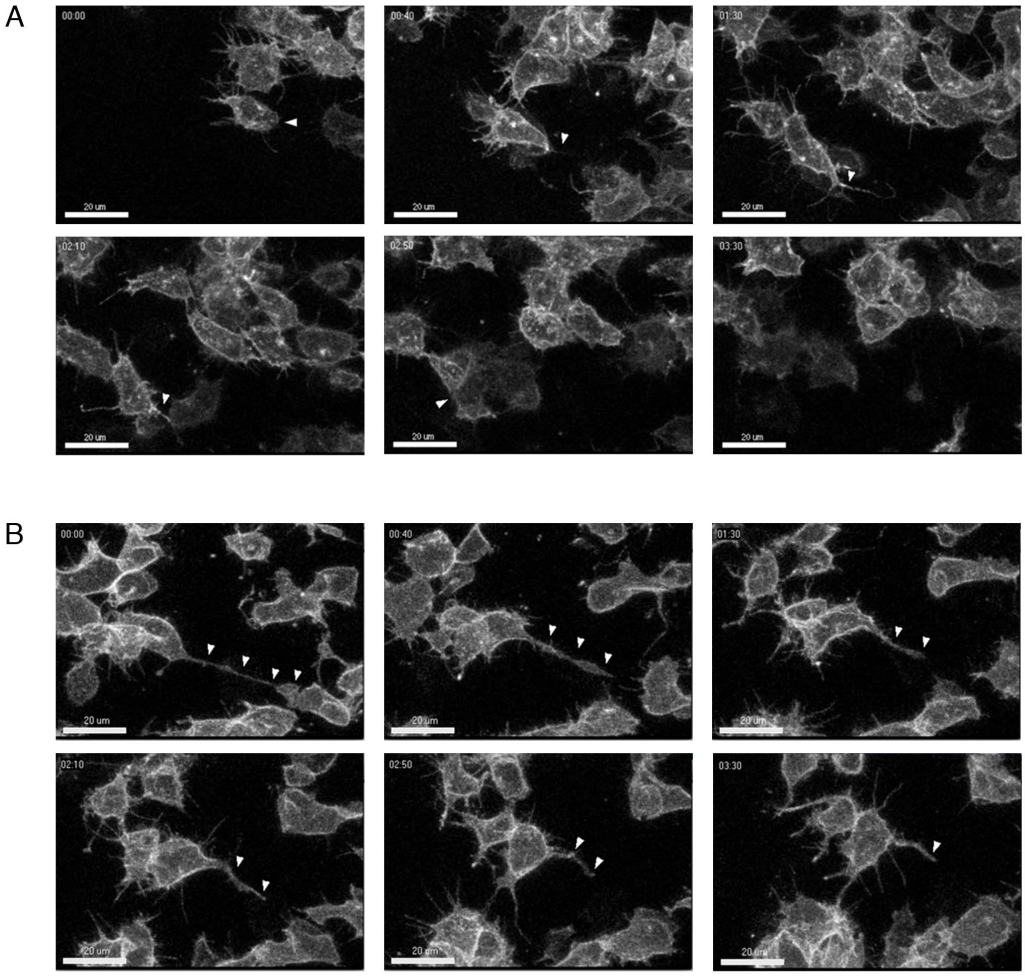


Figure 4.23 Altered mesoderm migration in *Crumbs2* mutants
Snapshots from time-lapse imaging of nascent mesoderm migration of wild type (A) and *Crumbs2* (B) mutants. Nascent mesoderm cells are very dynamic with filopodia and migrate away from the field in 3 hours. In Crumbs2 mutants, nascent mesoderm cells have long retraction tails that take about 3 hours to resolve. Cells start to form filopodia and begin migrating once these tails are resolved. Scale bar - 20 μm. Arrowheads point to retraction tails in wild type and *Crumbs2* mutants.

In addition to observing the behavior of epiblast cells at the streak, I also looked at the behavior of nascent mesoderm cells emergent from the primitive streak. Wild type cells are very dynamic with lot of filopodia and migrate quickly away from the streak in around 3 hours. In *Crumbs2* mutants, the nascent mesoderm cells have long retraction tails, probably a remnant of the long apically constricted cytoplasm of the cells. The nascent mesoderm cells take about 3-4 hours to resolve before this tail before forming filopodia and migrating away from the streak. This suggests that in addition to the inability of the cells to delaminate from the streak, the cells have an additional defect in reorganizing their cytoskeleton to facilitate cell migration. This could be a result of the altered cytoskeleton of the constricted cells at the streak with ectopic actin and myosin. These cells take longer to reorganize their cytoskeleton compared to wild type.

4.13. Discussion

Crumbs2 is required for efficient delamination at the primitive streak

Cell polarity remains largely unaffected in the epiblast in both the *Crumbs2* mutants and the complete loss of all forms of Crumbs. We therefore conclude that Crumbs proteins are not essential for the establishment of cell polarity in the early mouse embryo. Instead, the data suggest that Crumbs 2 is required to promote the EMT and to reinforce epithelial integrity in tissues undergoing dynamic processes such as EMT at the primitive streak.

The gastrulation defect in *Crumbs2* mutant embryos indicates that Crumbs2 is required for cell delamination during epithelial to mesenchymal transition at the primitive streak. Despite the basement membrane breakdown, they have a delay

in the down-regulation of adherens junctions and therefore majority of the cells at the streak remain Sox2 positive, i.e. epithelial. This is in contrast to other mutants like *Rac1*, which have a defect in mesoderm migration wherein the cells accumulated at the streak express Snail1 (Figure 4.9I). The absence of Crumbs2 makes the cells at the streak more adherent and less likely to delaminate. Loss of Crumbs in *Drosophila* ectoderm leads to a failure of Zonula adherens maturation, which leads to loss of adherens junctions and other apical proteins (Tepass, 1996). In contrast, loss of Crumbs2 in mice leads to a delay in E-cadherin down-regulation during EMT at the primitive streak (our study,(Xiao et al., 2011). We propose that Crumbs2 is required to make E-cadherin more dynamic in tissues undergoing morphogenesis. Crumbs was shown to regulate the endocytosis of Notch receptor and regulate membrane dynamics in *Drosophila* epithelia (Firmino et al., 2013; Richardson and Pichaud, 2010). In the primitive streak, Crumbs2 could regulate the endocytosis of E-cadherin, making it easier for cells to lose their adherens junction and therefore undergo EMT. In the absence of Crumbs, there could be a delay in E-cadherin down-regulation, which affects cell delamination.

Crumbs has been shown to regulate actin-myosin cytoskeleton in *Drosophila* trachea and salivary placode (Letizia et al., 2011; Roper, 2012). During EMT when cells delaminate, they also reorganize their actin-myosin cytoskeleton. *Crumbs2* mutants have defects in the reorganization of the actin-myosin cytoskeleton. We propose that Crumbs2 regulates this reorganization of the actin-myosin cytoskeleton and the absence of Crumbs2 leads to a delay or improper reorganization with cells accumulating at the streak. Additionally, the cytoskeletal reorganization defects could directly affect the dynamics of adherens

junctions. Alternatively, the regulation of adherens junctions and cytoskeletal reorganization could be two independent functions of Crumbs2.

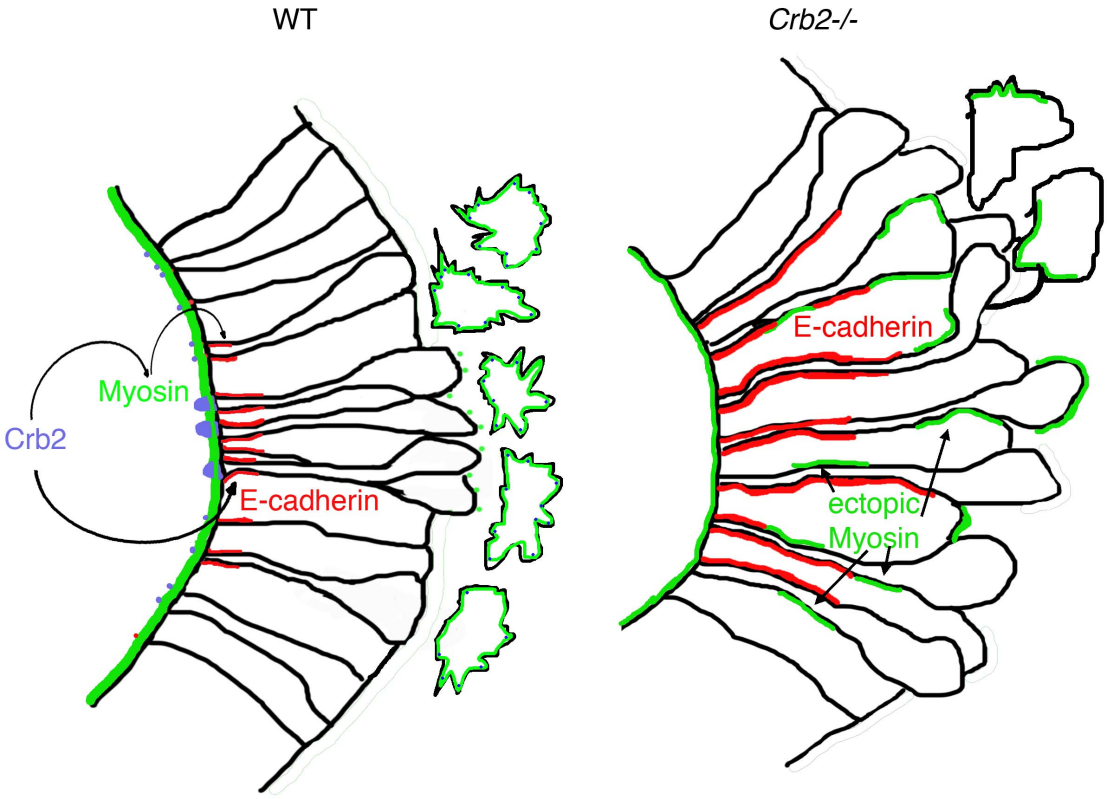


Figure 4.24 Model for Crumbs2 function at the primitive streak
In wild type, when epiblast cells reach the primitive streak, they constrict their apical membrane to adopt the bottle-neck shape. These constricted cells at the streak have enriched Crumbs2 expression in their apical membrane. This Crumbs2 is required to recruit apical myosin network for cell delamination. The presence of a myosin scaffold ensures that cells delaminate in an orderly manner and would also relieve the tension in the epithelium, as cells actively leave the epithelium. In addition, Crumbs2 regulates the reorganization of apical myosin to facilitate cell migration following EMT. By regulating actin-myosin

reorganization, Crumbs2 could indirectly affect the dynamics of the adherens junctions. Alternatively, Crumbs2 could directly affect the dynamics of the adherens junctions. In *Crumbs2* mutants, epiblast cells at the streak constrict their apical surface but fail to delaminate. The mutants have reduced apical myosin, ectopic myosin along the cells in the epiblast and fail to reorganize it efficiently. In addition to cytoskeletal defects, the constricted cells at the streak fail to down-regulate E-cadherin and therefore we see an accumulation of E-cadherin expressing cells at the primitive streak.

In contrast to Xiao, 2011, our results show that loss of a polarity regulator Crumbs2 does not lead to more cells delaminating from the epithelium as the accumulated cells at the primitive streak express Sox2, an epithelial marker and not Snail1, a mesodermal transcription factor. This suggests that during EMT losing polarity is not sufficient for cell delamination. Mutations in polarity proteins have been associated with tumors and metastatic cancers. These mutations were thought to promote EMT leading to tumor dissemination. Interestingly, recent studies have shown that cell junctions are required for tumor dissemination and loss of E-cadherin prevents this process (Shamir et al., 2014). The contribution of epithelial dynamics to the EMT process still remains largely unexplored.

Crumbs2 regulates epithelial integrity in dynamic epithelia

The primitive streak is a dynamic epithelium with cells constantly exchanging neighbors as cells delaminate from the epithelium. Our results suggest that the primitive streak is particularly sensitive to the loss of Crumbs2 function. Based on the cell shape changes we observed in the epiblast, we are able to rescue the epithelial instability in Crumbs2 mutants by preventing the formation of the primitive streak and subsequent cell delamination. During EMT, while the cells are undergoing the transition and gaining migratory machinery, they physically

delaminate from the epiblast epithelium. As a cell leaves the epithelium, the remaining cells reorganize themselves to fill in the void, in the process gaining new neighbors and re-establishing polarity. We speculate that this local rearrangement of cells to maintain the stability of the remaining epithelium at the primitive streak requires Crumbs2 function, and loss of Crumbs2 leads to local disorganization as cells delaminate. This function of Crumsb2 could be mediated by its recruitment of myosin. The presence of myosin scaffold at the primitive streak could relieve the tension in the epithelium as cells delaminate. It could function as a mechanism of communication between cells to facilitate local cell rearrangements. In the absence of the myosin scaffold, communication between cells is compromised therefore local cell arrangements would affect the stability of the epithelium.

Crumbs2 acts at the level of tissue integrity rather than cell polarity

Cell delamination during EMT can be regulated both cell autonomously and non-cell autonomously. Our data from the chimeric embryos suggests that Crumbs2 regulates cell delamination at the primitive streak non-cell autonomously. The process of cell delamination is highly coordinated and controlled as when cells leave the epithelium, new junctions are established in the remaining cells. Since both the processes seem to occur simultaneously, a defect in one of the steps could affect the other. An inability to establish new junctions and rearrange contacts might lead to a delay in cell delamination. This would suggest that when Crumbs2 is expressed at the primitive streak, cells could locally reorganize their junctions and therefore facilitate the delamination of mutant cells. Our results support the hypothesis that Crumbs2 is required to regulate local cell rearrangements to maintain the primitive streak epithelium while cells

delaminate. Our results throw light on a largely unexplored phase of EMT, i.e. maintenance of the epithelium.

Chapter 5 Interaction of Crumbs2 with other proteins

Introduction

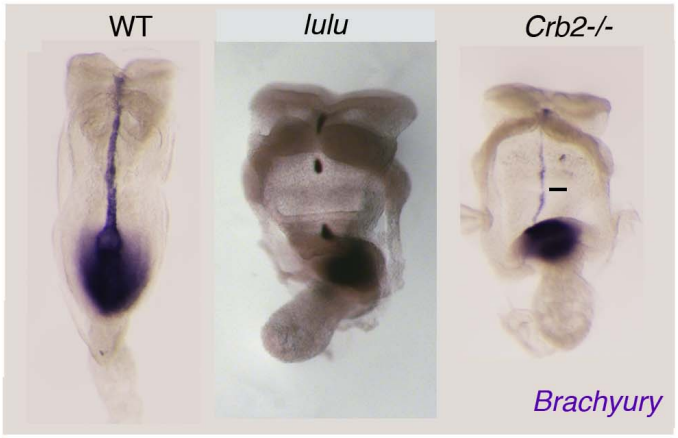
The intracellular domain of Crumbs besides recruiting the core Crumbs complex interacts transiently with a number of proteins in a cell type specific manner (Bulgakova and Knust, 2009). These transient interactions link Crumbs to other polarity regulators like Par complex and also link it to the cellular cytoskeleton and are therefore important for its function. Here I will describe the genetic interaction of Crumbs2 with Epb4.1l5, a FERM domain protein, Pten, a lipid phosphatase, and p120 catenin.

5.1 Crumbs2 and Ebp4.1l5

Ebp4.1l5 (also called YMO1) is the mammalian homolog of *Drosophila* Yurt, which was shown to physically interact with the intracellular domain of Crumbs. Yurt is expressed basolaterally in epithelia and was shown to negatively regulate the domain of Crumbs expression. However, Yurt transiently localizes to the apical domain in mid-to-late stage embryos and at the stalk membrane in PRCs and Crumbs mediates this apical recruitment of Yurt (Laprise et al., 2006). An ENU induced allele of Ebp4.1l5 called *lulu* has a gastrulation phenotype similar to *Crumbs2* mutants (Lee et al., 2007). These mutants have reduced mesoderm-derived tissues and fail to close their neural tube. Similar to Crumbs2 mutants, they have an accumulation of cells at the streak. I examined the *lulu* mutant primitive streak to determine the nature of cells accumulated at the primitive streak. I found that, similar to *Crumbs2* and *Poglut1*^{wsnp}, mutants, the primitive streak in *lulu* mutants was predominantly Sox2 expressing with Snail1 positive cells scattered between them. This suggests that at a cellular level, lulu and

Crumbs2 have similar phenotypes, suggesting that they could function together to regulate cell delamination.

A



B

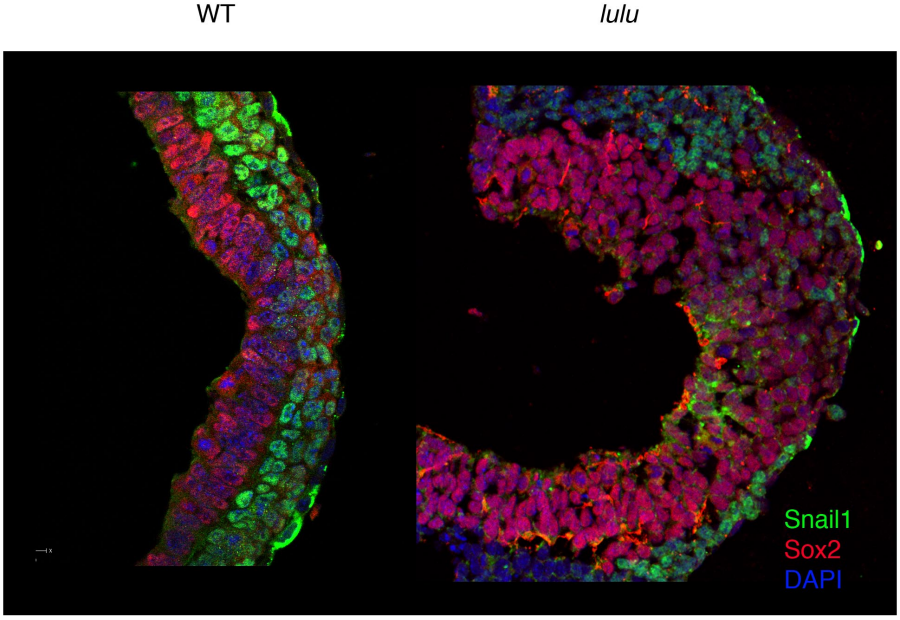


Figure 5.1 Cell delamination defect in *lulu* mutants
In situ for expression of *Brachyury* in wild type, *lulu* and *Crumbs2* mutants showing the discontinuous midline in the mutants at E8.5. (Dorsal view, anterior is up). Transverse sections through the primitive streak of wild type and *lulu* mutants immunostained for Snail1 and Sox2 (B). *lulu* mutants have predominantly Sox2 expressing cells at the streak and Snail1 positive cells scattered in between.

Lulu was thought to regulate actin-cytoskeletal reorganization during gastrulation EMT. The similarities in their phenotype led us to investigate the expression of these proteins in both the mutants. Lee et al. had shown that *lulu* mutants still have apical Crumbs using the pan-Crumbs antibody which recognizes all three mammalian Crumbs. To investigate whether Crumbs2 expression is specifically altered, we looked at the expression and localization of Crumbs2 in *lulu* mutants. Crumbs2 is enriched apically in the epiblast cells and is lost following EMT in the mesoderm cells. Both sections through the primitive streak and en face views suggest that apical membrane localization of Crumbs2 is not altered in *lulu* mutants (Figure 5.2). Additionally, Crumbs2 was properly down regulated as cells underwent EMT at the streak in *lulu* mutants.

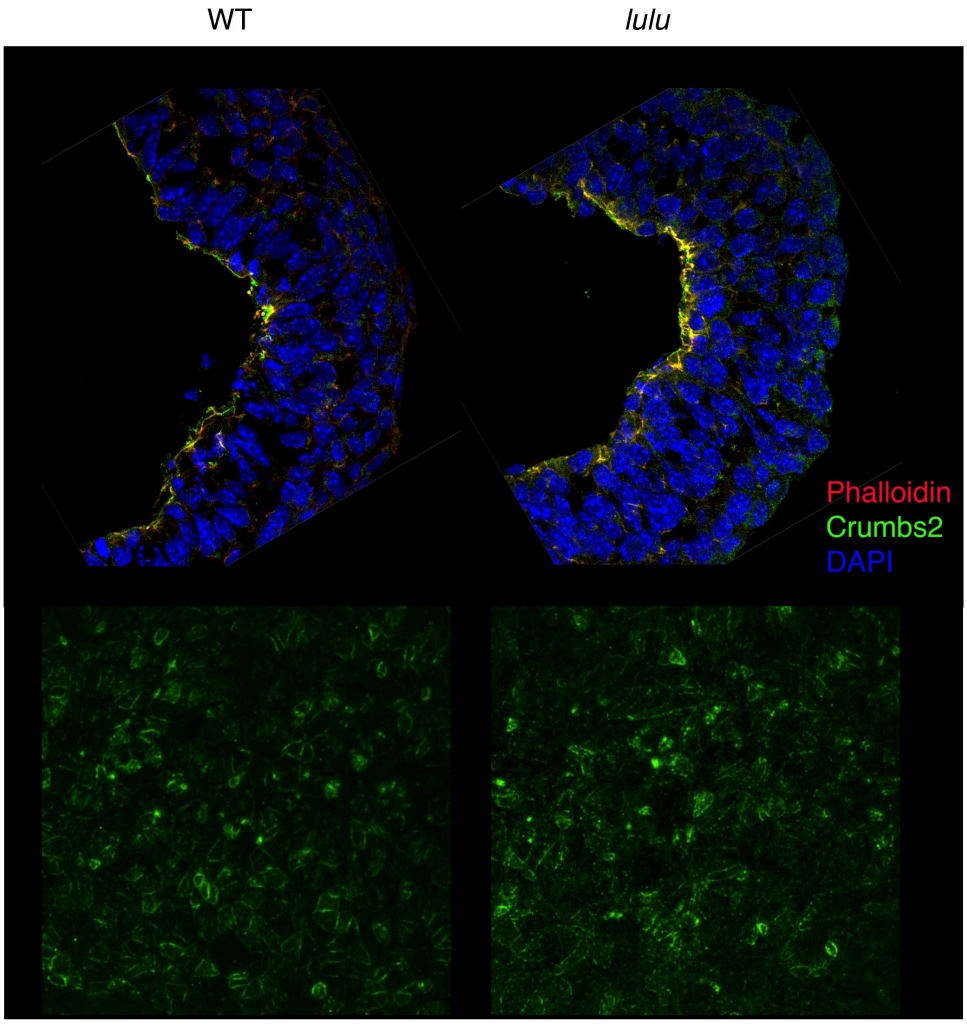


Figure 5.2 Crumbs2 localization in *lulu* mutants
Transverse section through the primitive streak of wild type and *lulu* mutants immunostained for Crumbs2 and phalloidin. Lower panel, enface view of the primitive streak immunostained for Crumbs2 expression at E7.75. Crumbs2 expression is not lost from the apical membrane in *lulu* mutants.

Since Crumbs2 expression was not altered in *lulu* mutants, we investigated the expression of lulu (YMO1) in *Crumbs2* and *Poglut1*^{wsnp} mutants.

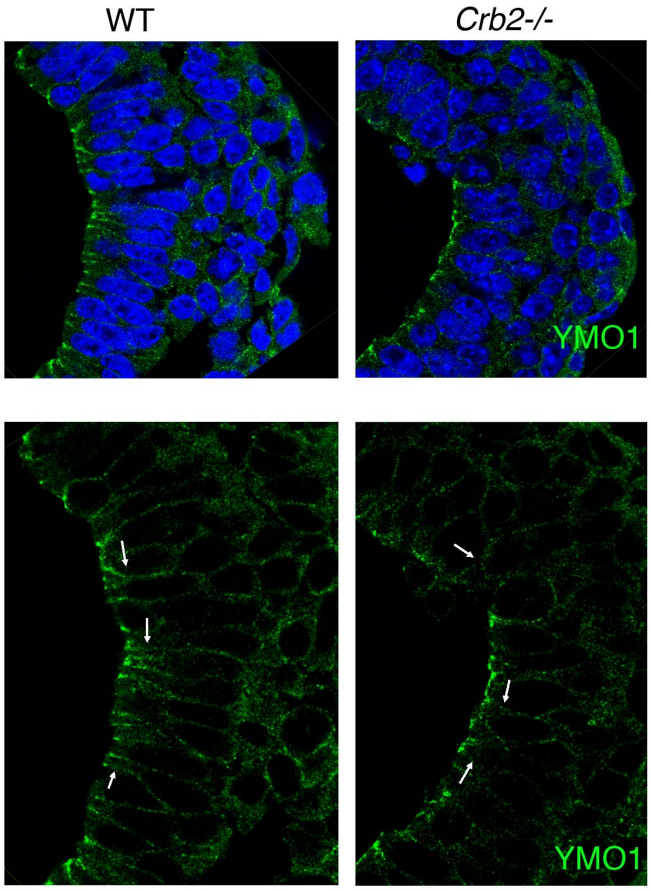


Figure 5.3 Lulu localization in *Crumbs2* mutants
Transverse sections through the primitive streak of wild type and *Crumbs2* mutants immunostained for YMO1 (or lulu) at E7.5. Lulu is enriched apically in the epiblast cells and it is reorganized as cells undergo EMT at the primitive streak in wild type and Crumbs2 mutants. Notice the lateral extension of Lulu localization in wild type (arrows). At the same distance from the apical surface in *Crumbs2* mutant shows lower levels of Lulu protein.

Lulu is enriched apically and reorganized as cells undergo EMT at the primitive streak. In *Crumbs2* mutants, the apical enrichment of Lulu/YMO1 was not altered, and Lulu was reorganized as cells underwent EMT at the streak. However, its apical domain of localization was slightly reduced in *Crumbs2* mutants (Figure

5.3). In wild type Lulu was enriched at the level of the adherens junctions and extends a little laterally. The domain of lateral expression was reduced in *Crumbs2* mutants (arrows in Figure 5.3). It was hard to determine if this reduction was primary or secondary due to change in the shape of the cells in *Crumbs2* mutants. Since the expression of neither protein was altered substantially in the corresponding other mutants we speculate that they do not affect each other's localization or reorganization during EMT. Instead, they function together as a complex to facilitate cell delamination, and each can be correctly localized independent of the other. Alternatively, they could be functioning in parallel pathways.

5.2 Crumbs2 and Pten

Crumbs maintains epithelial integrity by repressing Rac1 and phosphoinositide 3-kinase (PI3K) signaling. The cuticle defects in *Crumbs* mutants could be partially rescued by expressing a dominant negative form of *PI3K* or by also over-expressing *Pten*, a lipid phosphatase. The interaction between Crumbs and PI3K signaling maintains epithelial integrity and maintains the ratio between the apical and basolateral membrane in epithelial cells (Chartier et al., 2011).
We wanted to determine whether there was a genetic interaction between mammalian *Crumbs2* and *Pten*.

The epithelial instability in the Crumbs2 mutants is reflected in the shape of the cells in the neural epithelium. The cells in the neural epithelium of *Crumbs2* mutants have reduced apical surface and have expanded basolateral domain (Figure 4.11). Shortly after the basement membrane integrity is lost and cells lose

their epithelial contacts and start mixing with the mesoderm cells (Figure 4. 11H and (Xiao et al., 2011)).

Pten, a lipid phosphatase is also required for neural tube closure. Conditional deletion of *Pten* in the epiblast using the Sox2Cre generates mutants, which fail to close their anterior neural tube (Grego-bessa J). The neural epithelium in these mutants has multiple abnormal folds. A close examination of cell shape in the neural epithelium shows that the cells are shorter and have increased apical surface area. Since, the phenotypes of *Crumbs2* and *Pten* mutants appeared to be opposite, we wanted to see whether they function in a single pathway to regulate cell shape during neural tube closure. Interesting despite having the opposite phenotypes both *Crumbs2* and *Pten* single mutants fail to close their respective neural tubes suggesting that both extremes, constricted cells or expanded cells affect this process.

Using the conditional alleles and *Sox2Cre* transgene (Hayashi et al., 2002), we generated an epiblast deleted *Crumbs2 Pten* double mutant. The anterior neural epithelium of the double mutant resembled that of the *Pten* mutants, in that they had multiple abnormal folds. En face views with staining for ZO1 confirmed that the apical surface area in the double mutants was greater than the wild type and similar to the *Pten* single mutants (Figure 5.4C). Pten was epistatic by this assay. We wanted to determine whether the change in cell shape in *Crumbs2* mutant led to epithelial instability or was it a consequence of the same. To determine this, we examined the integrity of the neural epithelium in the epiblast deleted *Crumbs2 Pten* double mutants.

In sections of the neural epithelium, we found that cell mixing between epithelial and mesodermal cells still occurred in the double mutants (Figure 5.4D). *Crumbs2* was epistatic by this assay. This suggests that the cell shape changes in the *Crumbs2* neural epithelium are a consequence of the epithelial instability and not its cause.

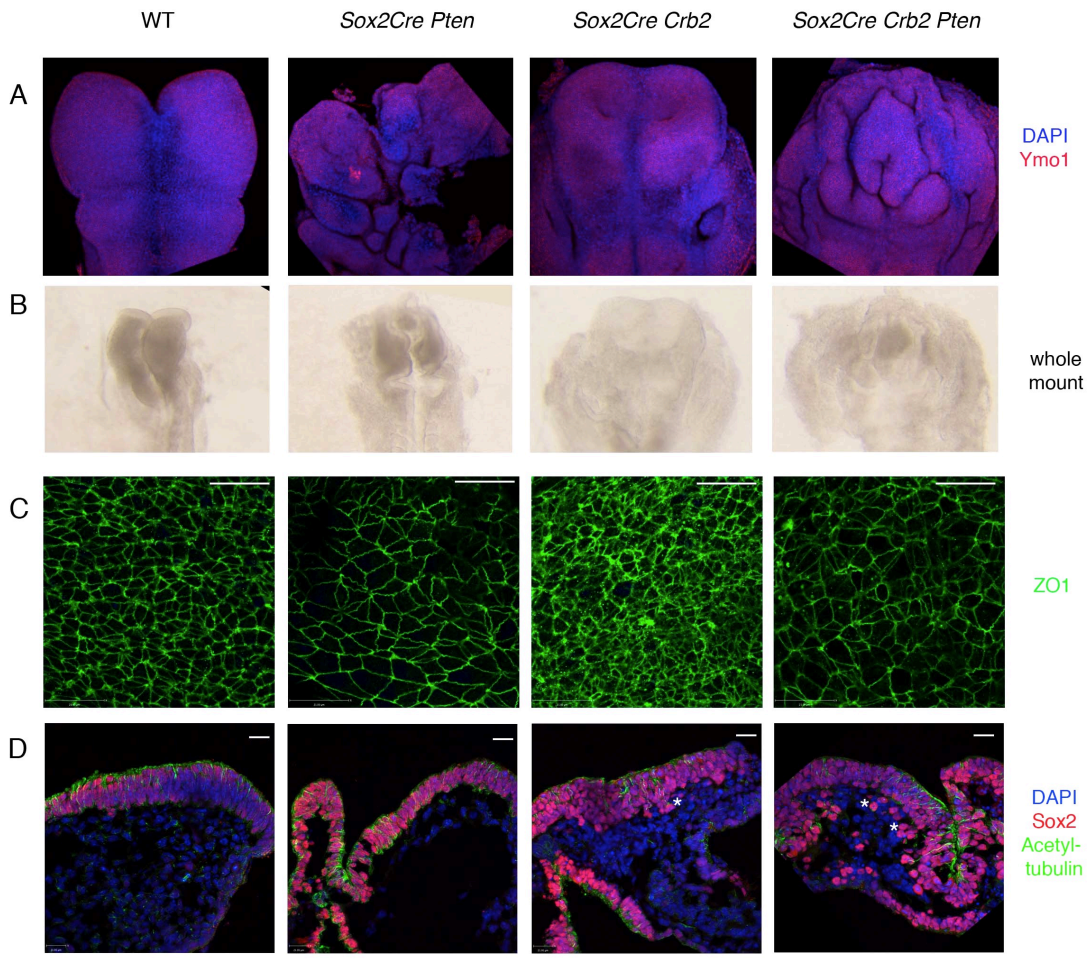


Figure 5.4 Crumbs2-Pten genetic interaction
Whole mount view of the neural epithelium of wild type, *Pten*, *Crumbs2* and double mutants and the corresponding embryos (A and B). En face view of the neural epithelium of wild type and mutants stained for ZO1 to look at the apical surface areas of the cells. The double mutants have cell surface area similar to

Pten mutants. Transverse sections through the neural epithelium of the wild type and the mutants at E8.0 immunostained with Sox2 (red) and acetylated tubulin (green). Notice the *Pten* mutants have thinner neural epithelium while *Crumbs2* mutants have thicker. The cell-mixing defect is much worse in the neural epithelium of double mutants, suggesting the cell shape changes are not responsible for epithelial instability. Asterisk indicates epithelial cells that have lost basement membrane but still express Sox2 and can mix with the underlying mesoderm cells.

5.3 Crumbs2 and p120 catenin.

p120 catenin is protein containing armadillo repeats which binds to E-cadherin. p120 catenin regulates the stability and turnover of adherens junctions. Epb4.1l5 can physically interact with p120 catenin and this interaction is important during its role in EMT (Hirano et al., 2008). Epb4.1l5 recruits p120 catenin from its cadherin complex and promotes the formation of a p120-paxillin complex, whose function is unknown. We wanted to determine whether p120 catenin is indeed downstream of Epb4.1l5. We obtained a conditional allele for *p120 catenin* and generated null alleles to determine the phenotype of mice lacking p120 catenin. We found that mice lacking p120 die at mid gestation (E9). They have a reduction in mesoderm-derived structures. Their neural tube has multiple abnormal folds and they fail to close their neural tube. Besides defects in gastrulation, mice lacking p120 catenin have additional phenotypes such as posterior axis duplications (Figure 5.5A). The embryos that did not have axis duplication morphologically resembled *lulu* and *Crumbs2* mutants as seen by *Brachyury* expression at E8.5 (Rocio Hernandez Martinez). They fail to elongate along the anterior-posterior axis and die by around E9 (Figure 5.5B).

The phenotype of p120 catenin knock out suggests that it has a role during gastrulation EMT. A further analysis of the phenotype would shed light if it functions together with Crumbs2 and Epb4.1l5 or independently during gastrulation EMT.

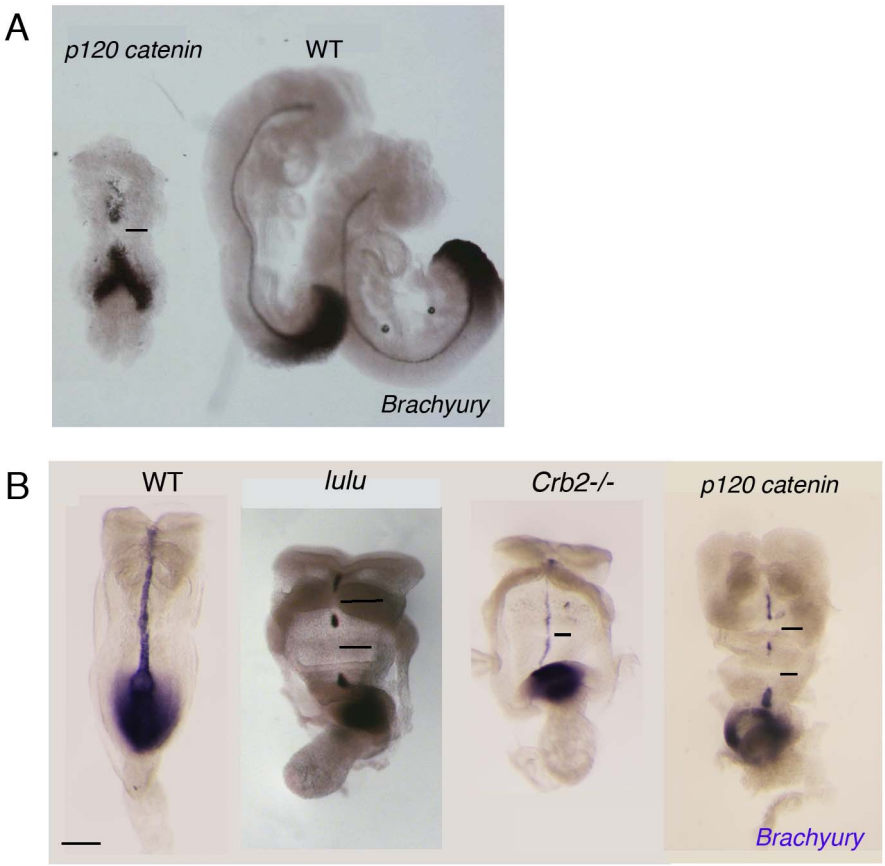


Figure 5.5 Phenotype of mice lacking *p120 catenin*
In situ for expression of *Brachyury* at E8.5, shows posterior axis duplication and discontinuous midline (A). These embryos are also shorter in along the anterior-posterior axis compared to wild type. Comparison of *Brachyury* expression in wild type, *lulu*, *Crumbs2* and *p120 catenin* mutants (B). The *p120 catenin* mutants that did not have axis duplication morphologically resembled both *lulu* and *Crumbs2.* They had a small primitive streak and discontinuous midline seen by *Brachyury* expression at E8.5. Dorsal view with anterior is on top. Scale bar- 150 µm. Bottom panel *Brachyury* in situ on *p120 catenin* mutant was performed by Rocio Hernandez.

Chapter 6 Closing remarks

6.1 Summary

The Epithelial to Mesenchymal transition is a complex multistep process that is tightly regulated. While canonical players have been established since this process was first described anatomically, our understanding of this process still remains in its nascent stages. With the help of mouse mutants, I have identified novel regulators of gastrulation EMT in mouse embryos. In this thesis I have described the role of Protein O-glucosyltransferase1, an enzyme that regulates the glycosylation of proteins in the ER during gastrulation. Although it is essential for Notch signaling, during gastrulation it is required for the apical membrane localization of Crumbs2. Secondly, I have shown that Crumbs2 is essential for efficient cell delamination at the primitive streak during gastrulation EMT. Both of these findings are important as they suggest novel but specific functions of these proteins which were previously uncharacterized.

6.2 Glycosylation of Crumbs2

In this thesis, I identified Crumbs2 as a novel biologically relevant target of Poglut1. This finding is particularly important because current studies always associate Poglut1 with Notch signaling because of reports in *Drosophila*. Here I have shown that mammalian Crumbs2 is glycosylated by Poglut1 and this is important for its apical membrane localization. This function of Poglut1 appears to be specific to mammals. Therefore studies with human mutations in Poglut1 must consider its role in Crumbs signaling besides Notch signaling.

This study gives some insight into the potential causes of human Retinitis pigmentosa. Mutations in human Crumbs proteins have been associated with Retinitis pigmentosa and other retinal degeneration. However since most of the mutations exist in the extracellular domain of Crumbs, its potential function or importance was unknown. Analysis of the human mutations shows that around 40 percent of the mutations are in the EGF repeats of *CRUMBS1*, while a few are directly in the glycosylation motif sequence (Bulgakova and Knust, 2009; den Hollander et al., 2004; den Hollander et al., 1999; Jalkh et al., 2014; Li et al., 2014). This would suggest that human mutations could alter the glycosylation status of Crumbs and therefore affect its membrane localization. From this study we know that membrane localization of Crumbs is essential for its function. Therefore these mutations could affect Crumbs functions. My data suggest that glycosylation of Crumbs2 is an additional layer of control to regulate its membrane localization during mammalian gastrulation.

6.3 Crumbs2 and its role in gastrulation

Crumbs was the first polarity protein to be identified in *Drosophila*. Since its discovery 25 years ago as an apical determinant, its canonical role as a polarity determinant is currently being challenged in many systems. The data in my thesis show that mammalian Crumbs are not essential for the establishment of epiblast polarity. Instead, my data with the *Wnt3* double mutants and conditional deletion of *Crumbs2* using *T-CRE*, suggests that Crumbs2 has a specific function at the primitive streak during mammalian gastrulation EMT.

Crumbs2 expression is enriched both transcriptionally and at the protein level at the primitive streak. This suggests that there might be a potential link between

the signaling pathways at the streak, which could enhance its expression at the streak to promote EMT. Fgf signaling in *Drosophila* eye regulates Crumbs expression as *Drosophila* mutant for either the ligand (*Branchless*) or receptor (*Breathless*) fail to express Crumbs (Mukherjee et al., 2012). Since the mouse mutant for Fgf signaling have an early gastrulation phenotype, it would be hard to determine whether Fgf signaling regulates Crumbs2 expression.

The regulation of cell delamination by Crumbs2 is an unexpected finding in itself. Naively loss of apical determinants should expedite the process of EMT as it would mean one less step. However, my data suggests that the process of EMT is more complex than assumed. Instead of cells expediting EMT, I see the reverse phenomenon: cells find it harder to do EMT in the absence of Crumbs. My data suggests that Crumbs2 has a specific role in regulating cell delamination during gastrulation EMT.

Unlike *Fgf8* mutants, which have an early gastrulation phenotype, *Crumbs2* mutants are indistinguishable from wild type during early gastrulation (E6.5 to E7.5). The phenotype of *Crumbs2* mutants becomes apparent only by head fold stages (E7.5). The relatively late appearance of the phenotype suggests that either Crumbs2 has a specific role in gastrulation EMT starting at the head fold stage or the defect in gastrulation is a result of slow accumulation of errors at the streak. The former case is particularly interesting as it suggests that EMT is regulated by different proteins, based on context. There appears to be a transition in some of the proteins expressed at the streak from early gastrulation to the head fold stage. *Eomesodermin*, a T-box transcription factor required for EMT and specification of cardiac mesoderm, is expressed in the streak from the

initiation of gastrulation until the early head fold stage. Likewise, *Mesp1* and *Mesp2* also required for gastrulation EMT are expressed during early gastrulation while the cardiac mesoderm are specified, and then turned off. I speculate that these additional transcription factors assist with the down-regulation of E-cadherin during early gastrulation and therefore we do not see an early gastrulation phenotype in *Crumbs2* mutants.

The phenotype of *Crumbs2* mutants suggests that Crumbs2 could be functioning at multiple levels to regulate EMT. The interaction of Crumbs2 with Epb4.1l5 through the FERM binding domain could regulate the recruit of apical Myosin to facilitate apical constriction and cytoskeletal reorganization for cell delamination. In addition to Epb4.1l5, the FERM binding domain of Crumbs2 can recruit aPKC, and together, they could organize this apical myosin scaffold for cell delamination.

Apart from cell delamination, Crumbs2 switches the cells from cell-cell interaction to cell-ECM interaction by recruiting Epb4.1l5 to the apical domain. The apical recruitment of Epb4.1l5 can facilitate the interaction of Epb4.1l5 with p120-catenin, displacing it from E-cadherin and destabilizing adherens junctions. The Epb4.1l5-p120 catenin complex can also bind to paxillin and this promotes its interaction with integrin.

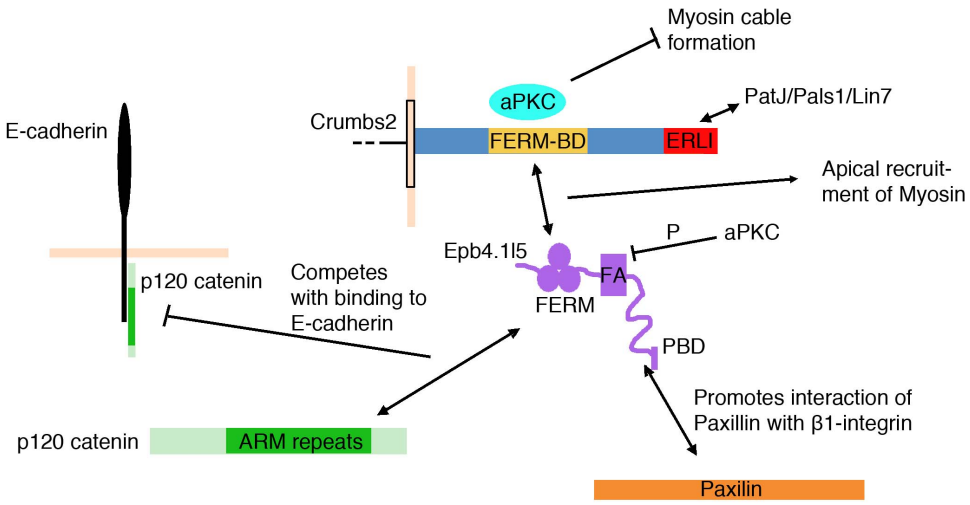


Figure 6.1 Crumbs2 function during gastrulation EMT
The intracellular domain of Crumbs2 has two domains, FERM binding domain and ERLI domain. The ERLI domain recruits the Crumbs complex (Pals1, PatJ and Lin7). The FERM binding domain of Crumbs2 recruits Epb4.1l5, a FERM domain protein. Together they regulate the apical recruitment of Myosin for apical constriction and cytoskeletal reorganization to facilitate cell delamination at the primitive streak. By recruiting Epb4.1l5 to the apical domain, Crumbs2 promotes the destabilization of adherens junction and enhances the cell-ECM interaction. The FERM domain of Epb4.1l5 can bind to the armadillo repeats of p120 catenin. This interaction reduces the association of p120 catenin with E-cadherin and destabilizes it. Epb4.1l5 can also bind to paxillin through its C-terminus. This interaction enhances the association of paxillin to integrin and promotes the formation of focal adhesions. The function of Epb4.1l5 is negatively regulated by aPKC. The FERM binding domain of Crumbs2 can also recruit aPKC to the apical domain. Together, they can regulate the formation of organized myosin cables on the apical surface of the primitive streak to facilitate cell delamination in an orderly manner.

An aspect of EMT that my data has shed light on is the contribution of epithelial dynamics and cell communication in this process. Primitive streak is a 3-dimensional field of cells stretching from the proximal to distal tip to the embryo. The basement membrane breakdown is localized to around 5-6 cells wide. Cell delamination is tightly regulated and occurs as single cells leaving the epithelium. When a cell leaves an epithelium, it leaves behind a void that has to be quickly filled in by its neighbors. An interesting question is that in a 3-dimensional field of cells; how to do the cells decide which cells have to delaminate and which cells do not and what are the forces and or signaling mechanisms that communicate this information. Both these concepts suggest the importance of cell communication in regulating an organized EMT.

The existence of a myosin network at the apical surface of the epithelium suggests this actin-myosin scaffold might regulate that cell delamination. The scaffold could function as a sensor, where tension generated from cells leaving the epithelium is transmitted to its neighbors to facilitate cell arrangements. Secondly, the myosin scaffold could lead to formation of thermodynamically unstable organization. The resolution of these unstable organizations could provide a driving force in cell delamination. Additionally, formation of these unstable organizations would determine which cells delaminate in a 3-dimensional field of cells. The presence of such rosettes has been observed in the avian primitive streak (Wagstaff et al., 2008).

Two recent reports using live imaging analysis of *Drosophila* neuroblast delamination suggest a major role for myosin in organizing the delamination in a

planar polarized manner. Given the disorganization of this apical actin-myosin scaffold in Crumbs2 mutants, I speculate that Crumbs2 plays a major role in organizing this scaffold.

Given the 3-dimensional nature of gastrulation EMT, imaging this process live using fluorescent reporters in the mouse embryos will shed light on the sequence of events that lead to cell delamination during gastrulation EMT.

Chapter 7 Materials and methods

Mouse strains used and their PCR conditions

For routine genotyping, ear punches, yolk sacs or whole embryos were lysed in a proteinase K solution (100 µg/ml proteinase K) at 55°C overnight. The proteinase K was inactivated by boiling for 10 minutes at 90°C. The samples were diluted 1 to 10 and 5µl of the sample was mixed with an equal volume of a master PCR mix.

Mouse strains used:

Poglut1^{wsnp}, *Nodal-LacZ*, *TOPGAL*, knock-out first allele from Eucomm *Poglut1*^{gt}, conditional allele (*Poglut1*^{flox}) generated by crossing *Poglut1*^{gt} to actin-Flip mice, null allele (*Poglut1*$^{\Delta}$) generated by crossing conditional allele to *CAG-Cre*. *NICD-Rosa,* conditional allele of Crumbs2 (*Crumbs2*^{flox}), null allele (*Crumbs2-/-*) was generated by crossing the conditional allele to *CAG-Cre*, *Crumbs1*^{rd8}, *Crumbs3-/-*, *Wnt3-/-*, Rac1 conditional allele (*Rac1*^{flox}), *X-linked GFP, GFP-GPI, mT-mG, Brachyury-Cre*, *Sox2-Cre*, *CAG-Cre*, *EIIA-Cre*, *Epb4.1l5*^{lulu}, conditional allele of p120 catenin (*p120catenin*^{flox}), null allele of p120 catenin (*p120 catenin-/-),* conditional allele of Pten (*Pten*^{flox}).

Poglut1^{wsnp}

wsnp was generated by ENU mutagenesis of C57/BL6J mice. This allele harbors a T to C transition in the splice donor site of intron 3 of Poglut1. This change creates an SfaNI restriction fragment length polymorphism when assayed in genomic DNA using the allele-specific primer:

wsnpF- 5'-GTTCCTATCCCCTGCTCTCC-3'

wsnpR- 5'-GGACAGAGCCTAAGCCATCA-3'

Use PlatinumTaq, 0.08 µm per reaction and regular cycle with annealing at 55°C for 35 cycles. The PCR product was then digested with SfaN1 (1.5 units per reaction) at 37°C for 3 hours, and separated on 2% agarose gel with the following expected results:

wsnp mutant - 284 and 146 bp

WT - 430 bp

Poglut1^{gt}, *Poglut1*^{flox} and *Poglut1*$^{\Delta}$

The knock out first Poglut1 allele was obtained from Eucomm. The *Poglut1*^{gt} allele was identified by PCR for LacZ coding region (Jackson Labs).

The mice were crossed to *Actin-Flip* (Rodriguez et al., 2000) to remove the gene trap cassette. This generated the conditional allele (*Poglut1*^{flox}). These mice were then crossed to *CAG-Cre* (Sakai and Miyazaki, 1997) to generate the null allele. Allele specific primers were designed to determine the conditional and null alleles. The corresponding product sizes are mentioned.

Poglut1-Frt-lox2-F	5'-TGAGGGCAGTGATCCTTTTC-3'	wt-124
Poglut1-Frt-lox2-R	5'-CTCACCCACAGACACCGTTA-3'	mut-294
Poglut1-loxp3-F	5'-GGCCTCTGTGTCCAAAGGTA-3'	wt-197
Poglut1-loxp3-R	5'-TGCCTGGGATACAGACATTG-3'	mut-206
Poglut1-frt1-Lox3-F	5'-TGAGGGCAGTGATCCTTTTC-3'	wt-908
Poglut1-frt1-lox3-R	5'-TGGTACCCAGCACTATGGCTA-3'	flp+cre - 299

Crumbs2^{flox}

The conditional mouse lines were obtained from Jan Wijnholds. The following primers were used for genotyping the conditional allele.

HA7 forward 5'-TGCATCTTCTGAGATCAGGTG-3'

HA8 reverse 5'-ACCTGCCAGACTTCTCCTAC-3'

Products size - 303 bp for conditional (Floxed) and 106 bp for WT.

HA11 forward 5'-TGGAGATGGACAGTGTCCTC-3',

HA12 reverse 5'-GCTCTGGAAACAGTCTCCTTG-3'

Product size - 217 bp for conditional (Floxed) and 185 bp for WT.

The PCRs were carried out with annealing temperature at 58°C and 35 cycles. The PCR products were separated on a 1% gel.

Crumbs2-/-

Two separate PCR reactions were carried out.

HA7 and HA12 primers with annealing temperature at 55°C and gave a 140 bp product for null allele and no product for wild type.

HA7 and HA8 pair of HA11 and HA12 pair with annealing temperature at 58°C to give 106 bp or 185 bp for wild type respectively.

Crumb1^{rd8}

This allele was obtained from Jackson Laboratory. It harbors a single base pair deletion at nt3481. This mutation causes a frameshift and leads to a premature stop codon before the transmembrane domain of Crumbs1. The PCR conditions and allele specific primers were used as published (Mehalow et al., 2003).

Crumbs3-/-

Frozen sperm was obtained from lexicon genetics. The lines were re-derived by in vitro fertilization (performed by the mouse transgenic core facility). Allele specific primers were used for genotyping.

Wild type F- 5'-GAAATTGATAGGGACAATAAAGG-3'
Common R- 5'-CAACCTCGAGTTGCTGATCTG-3'
Mut F- 5'-GCAGCGCATCGCCTTCTATC-3'

Following PCR with annealing at 55°C for 35 cycles, the PCR products of 285bp (wild type) and 325 bp (mutant) respectively were separated on a 2% agarose gel.

Epb4.1l5^{lulu}

lulu was generated from an ENU-mutagenesis screen. The *lulu* mutation destroys the BsaJ1 enzyme site and creates a restriction fragment length polymorphism. Primers and PCR conditions used were same as published (Lee et al., 2007).

Pten^{flox} and *Pten*$^{flox/\Delta}$

The conditional lines were obtained form Pier Paolo Pandolfi. The genotyping protocol and PCR primers were same as published (Trotman et al., 2003).

Nodal-lacZ

The *nodal-lacZ* knock-in allele (Collignon et al., 1996) was a gift from Elizabeth Robertson (University of Oxford, Oxford) and the PCR for genotyping was as published.

TOPGAL

TOPGAL mice were provided by Elaine Fuchs (Rockefeller University, New York) and genotyped by PCR using primers for the LacZ coding regions (DasGupta and Fuchs, 1999).

p120 catenin

The conditional allele of *p120 catenin* was obtained from Elaine Fuchs lab (Rockefeller University, New York). These mice were crossed with *CAG-Cre* to generate the null allele. The primers and PCR conditions for genotyping were as published (Perez-Moreno et al., 2006).

Rac1^{flox}

The conditional allele was a gift from Victor Tybulewicz. The PCR primers and conditions used were as published (Walmsley et al., 2003).

Wnt3

The conditional allele was obtained from Jaime Rivera Perez (University of Massachusetts medical school, Worcester). Crossing the conditional allele to *Sox2-Cre* generated the null allele. The PCR conditions and primers were used as published (Tortelote et al., 2013).

Brachyury-Cre

The mice were obtained from Mark Lewandoski (National Cancer Institute, Frederick). Mice carrying the Cre transgene were genotyped using the standard Jackson labs Cre geneotyping protocol (Perantoni et al., 2005).

NICD-Rosa, Sox2-Cre, mTmG, EIIA-Cre were obtained from Jackson Laboratories and were genotyped as per the protocols.

X-link GFP and *GPI-GFP* mice were a gift from Kat Hadjantonakis (Sloan Kettering, New York). Mice were genotyped by GFP fluorescence.

ENU mutagenesis screen and mapping of *wsnp*

wsnp allele was isolated from an ENU-induced mutagenesis screen carried out in the lab (Kasarskis et al., 1998). Jeffrey D. Lee mapped the mutation to a 407 kb interval between D16Mit90 and D16Mit12 simple sequence length polymorphism (SSLP) markers using classic recombination. Genomic DNA was purified from three mutants pooled together. DNA across the genomic interval was enriched using Agilent SureSelect solution based technology with a custom target capture. Samples were multiplexed and bar-coded for SOLiD sequencing. Sequencing reads were aligned to the C57BL/6 reference genome using SHRiMP.

Phenotypic analysis

Whole mount in situ hybridization and LacZ stainings were performed as published (Lee et al., 2007). The following probes were used: *Mox1, T, Tbx6, Nkx2.5, Uncx4.1, Hes5, Hes7, Lunatic Fringe and Delta-like 1*.

Immunostaining

Embryos were dissected in ice cold PBS-BSA and fixed in 4 percent PFA for one hour at room temperature. They were washed in ice cold PBS three times and equilibrated in 30 percent sucrose for 2-3 hours and then embedded in OCT (optimal cutting temperature). Immuno staining on frozen sections were carried out as published (Lee et al., 2007). Briefly, sections were washed in PBS three times, incubated in blocking buffer (1% Heat inactivated donkey or goat serum, 0.1% triton X-100 in 1XPBS) for 1-2 hours. Primary antibodies were diluted in blocking buffer and sections were incubated over night at 4°C. The secondary antibodies were diluted in blocking buffer and incubated for 1 hour at room temperature. For whole mount immunostaining, following fixation in 4 percent PFA for one hour, embryos were washed in ice cold PBS and incubated in blocking buffer overnight at 4°C. The primary antibody was diluted in blocking buffer and the embryos were incubated overnight in primary. They were washed three times in ice cold PBS, and 3 more times every 2 hours and incubated in secondary antibody over night at 4°C. Following extensive washes, embryos were flat mounted on a slide for en face imaging or mounted in glass bottom plates for imaging. Rhodamine-conjugated phalloidin at 10 U/ml and DAPI were included in the secondary incubation.

The following were the antibodies used which were generated in labs : anti-Crumbs1 and anti-Crumbs2 were obtained from Jane McGlade (Hospital for Sick Children, Toronto) and were used at 1:100 and 1:50 respectively. Anti-pan-Crumbs was a kind gift from Ben Margolis (University of Michigan, Ann Arbor) and was used at 1:200. Anti –Snail1 was a gift from Antonio García de Herreros (University of Pompeu Fabra, Barcelona) and was used at 1:100 and anti-PatJ was

a gift from André Le Bivic (Developmental Biology Institute of Marseille, Marseille) and was used at 1:200. Anti-Ymo1 (Lee et al., 2007) and anti-Arl13b (Caspary et al., 2007) were used at 1:200. Commercially available antibodies used were as follows: Anti-E-cadherin (Sigma 1:200), anti-laminin (Sigma 1:200), anti-Myosin IIB heavy chain (Covance 1:400), anti-Sox2 (Santa Cruz 1:100), anti-ZO1 (Zymed 1:200), anti-PHH3 (Upstate 1:400), anti-cleaved-caspase3 (Promega, 1:300), anti-GM130 (BD Biosciences 1:200), anti-Par3 (Upstate 1:200), anti-acetylated α-tubulin (Sigma 1:1000), anti-γ tubulin (Sigma 1:500), anti-pERM (Cell Signaling 1:100), anti- aPKC (BD Biosciences 1:100), anti-Tuj1 (Covance 1:1000), anti-Cleaved Notch1 (Cell Signaling 1:1000), anti-Notch1 (Abcam 1:200), anti-Brachyury (R&D 1:200), anti-Nanog (CosmoBio 1:500), phosphor-Myosin light chain2 (Cell signaling 1:200), anti β-catenin (BD Biosciences 1:200) and anti-V5 (Invitrogen 1:1000).

Whole mount cleaved Notch1 immunostaining

Embryos were dissected in ice cold PBS-BSA and fixed overnight in 4% PFA/PBS at 4°C, dehydrated in methanol and stored at -20°C overnight. Following rehydration, antigen unmasking was performed by placing the embryos in Vector unmasking solution (H-3300 Vector labs) at 98°C for 10 minutes. After reaching room temperature embryos were washed in milliQ water and kept in acetone at -20°C for 8 minutes. Following this the embryos were washed and incubated in blocking buffer overnight at 4°C (Blocking buffer -10% goat serum, 5% BSA, 0.3% Triton-X100 in PBS). The embryos were incubated in anti-cleaved Notch1 antibody (1:1000) for 2 days. Following 4-5 washes with blocking buffer, embryos were incubated in secondary antibody overnight at 4°C. The embryos

were washed extensively before mounting in glass-bottom dishes for confocal imaging.

Western blot analysis

Embryos were dissected in ice cold PBS and snap frozen immediately using dry ice. The embryos were lysed in RIPA buffer with protease inhibitor tablet. The lysates were left on ice for 20 minutes to equilibrate. Following this, they were subjected to sonication (3 X 30 seconds each). Lysates were incubated for 10 minutes in ice and then centrifuged to remove the cell debris. The supernatant was mixed with 2X SDS loading dye (1:1) and loaded on SDS-PAGE for analysis. The dilutions for primary antibody were as follows: anti-pan-Crumbs (Ben Margolis 1:2000), anti-Cleaved-Notch1 (Cell Signaling 1:1000), anti-Notch1 (Abcam 1:1000), anti-YAP (Cell Signaling 1:1000), anti-p-YAP (Cell signaling 1:1000), anti-GAPDH (Sigma 1:2000) and anti-V5 (Invitrogen 1:5000).

Cloning of Crumbs2

cDNA was synthesized from wild type embryos harvested at E8.5. Full length Crumbs2 was cloned into the gateway vector pDEST40 to generate Crumbs2 with His and V5 tag at its C-terminal. This was then sub-cloned with the tags into pCAGG vector to generate pCAGG-Crumbs2 for high expression in embryonic stem cells.

Generation of ES cell lines expressing full length tagged Crumbs2

ES cells were derived using the 2i protocol from both wild type and *Poglut1*^{wsnp} blastocysts (Silva et al., 2008) and eventually weaned off iMEFs. To generate stable cells lines expressing tagged full length Crumb2, wild type and *Poglut1*^{wsnp}

ES cells were electroporated with the pCAGG-Crumbs2 linearized by cutting at ScaI along with a circular *PGK-Puro-pA* plasmid (Tucker et al., 1996) that confers a transient puromycin resistance. Stable lines were selected using puromycin using published protocols (Hadjantonakis et al., 1998). 15 different colonies were screened for expression of tagged full length Crumbs2 by both immunofluorescence and western blots analysis with anti-V5 antibody. One cell line was selected for each wild type and *Poglut1*^{wsnp} based on high expression levels and was used for protein purification.

Embryoid Body differentiation

ES cells were trypsinized and re-suspended as 10^6 ES cells in non-adherent conditions (coated with Sigma coat) in 10-cm Petri dishes (VWR). EBs were cultured in LIF-free medium containing 10% FBS. Medium was replaced every day. The EBs were harvested from day 1 to day 6 to determine endogenous Crumbs2 expression. For protein purification, EBs from wild type and *Poglut1*^{wsnp} were harvested at day 2.

Protein purification

ES cells were cultured in large scale (6X15 cm plates). These ES cells were differentiated to embryoid bodies. Embryoid bodies were harvested from wild type and *Poglut1*^{wsnp} mutant at day 2, post induction of differentiation from stem cells and snap frozen. These were re-suspended in RIPA buffer with 2% DDM (*n*-Dodecyl β-D-maltoside) and Roche protease inhibitor tablet. They were subjected to three cycles of freeze-thaw to increase the release of membrane protein. Following this they were sonicated for 4 times, 30 seconds each. They were incubated at 4°C for one hour with continuous shaking. The lysates were clarified

by centrifugation for 20 minutes at 12,000rpm. The supernatant was incubated with anti-V5 antibody (1:1000) for 4 hours at 4°C shaking. This mixture was incubated with magnetic nickel beads (Invitrogen) for 2 hours at 4°C shaking. The beads were rinsed four times in RIPA lysis buffer and heating the beads in 2XSDS loading buffer at 95°C eluted the protein. The eluted protein was run on a 7% SDS-PAGE. The gel was washed 3 times in HPLC purified water and then stained with Pierce gel code blue stain reagent for 5-10 minutes. Following staining, the bands were cut out, subjected to chymotrypsin digest and used for mass spectrometric analysis.

N2B27 differentiation

To differentiate ES cells into neuronal lineage, ES cells weaned off iMEFs were plated at low density (10^4 cells per centimeter square) in gelatin-coated dishes. The following day media was changed to serum-free N2B27 media (neurobasal media with N2 and B27 supplement) as per established protocols (Boroviak and Rashbass, 2011). The cells were harvested at day 1 and day 10 and probed by immunofluorescence for differentiation into neural lineage.

Drosophila cuticle preparations

For cuticle preparations, embryos were collected on apple juice agar plates, washed with 0.7% NaCl and 0.1% Triton X-100, and bleached to remove the chorion. Embryos were fixed for 1 hour at 65°C in 1:4 glycerol : acetic acid and mounted in Hoyer's medium.

Scanning Electron Microscopy

E7.75 embryos dissected in ice cold PBS and were fixed with 2% PFA and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer. Following fixation, embryos were dissected to expose the primitive streak in 0.1 M cacodylate buffer and dehydrated in ethanol (Migeotte et al., 2011). Scanning electron microscopy was performed using a field emission microscope (Supra 25; Carl Zeiss), and images were acquired with SmartSEM (Carl Zeiss).

Generation of Chimeras

Mice carrying both *GPI-GFP* transgene (Rhee et al., 2006)and a deleted allele of *Crumbs2* were generated. Blastocysts generated from the intercross between these mice were harvested. ES cells were generated from GFP positive blastocysts using the 2i+LIF method (Silva et al., 2008). The GFP labeled wild type and *Crumbs2* mutant ES cells were independently injected into wild type blastocysts. These were implanted into pseudo pregnant females. These mice were harvested between E9.5 to E10.5 (equivalent to E8.5 to E9.5 normal development as chimeras are delayed). A total of 40 chimeras were analyzed. The endogenous GFP was used for the identification of *Crumbs2* mutant cells in the chimera analysis.

Image acquisition

Confocal microscopy was performed by using Leica-Inverted SP5 or Leica-Upright SP5 laser, point-scanning confocal microscope.

Data quantification and statistical analysis

Single cells were selected and their areas were determined using Volocity software. The width of the neural epithelium was also determined using Volocity. Average area of cells and width of the neural epithelium was calculated using Prism and student t-test was used for statistical analysis. The error bars indicate standard deviation or standard error.

Culturing mouse embryos for live imaging

Embryos were dissection with their yolk sac and ectoplacental cone intact in media maintained at 37°C (DMEM/F12 with 10% FBS). The embryos were positioned with the posterior side facing the objective into a hole created in a freshly prepared collagen matrix (BD Biosciences) on a glass bottom 35-mm Matek dish equilibrated with dissection media. The embryos were cultured in rat serum and imaged for 4-6 hours with frames taken every 8-10 minutes on a Leica SP5 or SP8 confocal microscope equipped with an incubation chamber maintained at 37°C and 5% CO2. The time-lapse movies were analyzed using Imaris and Metamorph software.

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